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#8

PATENT

ATTORNEY DOCKET NO. 08269/003001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Deggerdal et al.
Serial No.: 08/849,686
Filed : August 21, 1997
Title : ISOLATION OF NUCLEIC ACID

Art Unit: 1211
Examiner: Gary Kunz

6-3-98

Assistant Commissioner for Patents
Washington, DC 20231

DECLARATION OF [NAME] UNDER 37 C.F.R. § 1.131

I, ARNE DEGGERDAL [name], of ASKER, NORWAY [~~city~~, country], hereby declare:

1. I have held the position of RESEARCHER [~~title~~] at Dynal AS, Oslo, Norway since 1992 [year].

2. Dynal AS, established in 1986 [year], is a [~~publically traded or~~ privately held?] company which develops and markets magnetic tools for use in molecular and cellular biological assays.

3. Dynabeads® are a type of uniform, superparamagnetic polymer beads manufactured and marketed by Dynal AS. Dynabeads® were first offered for sale in the United States in Aug [month], 1986 [~~year~~]. Attached hereto as Exhibit A is a copy of the manufacturer's instructions provided to U.S. users of this product.

4. Dynabeads DNA DIRECT™ are also a type of uniform, superparamagnetic polymer beads manufactured and marketed by Dynal AS. Dynabeads DNA DIRECT™ are supplied as part of a ready-to-use kit designed for the easy and rapid isolation of nucleic acids from a liquid sample. The Dynabeads DNA DIRECT™ kit

Date of Deposit May 19, 1998
I hereby certify under 37 CFR 1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Lisa G Gray
Lisa G Gray

includes Dynabeads DNA DIRECT™ supplied in a cell lysis buffer, a washing buffer, and a resuspension buffer. The kit was first offered for sale in the United States in June, 1995 [~~month~~, year]. Attached hereto as Exhibit B is a copy of the manufacturer's instructions provided to U.S. users of this kit.

5. All statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: 05.05.98

Arne Dæggerdal
[insert name here; sign above]
Arne Dæggerdal

[insert company address, phone and fax numbers]
298922.B11

Dynal AS
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GERMANY/AUSTRIA: DEUTSCHE DYNAL GmbH
Postf... 19 65, D-20419 Hamburg
TEL: (... 36 68 11 FAX: (040) 36 60 40

UK/IRELAND: DYNAL (UK) LTD.
10 Thursby Rd, Croft Business Park, Bromborough,
Wirral, Merseyside L62 3PW
TEL: (0151) 346 1234 Fax: (0151) 346 1223

JAPAN: NIHON DYNAL K.K.
Champion Bldg., 1-1-9, Atago, Minato-Ku, Tokyo 105
TEL: (03) 3435-1558 FAX: (03) 3435-1526

USA/CANADA: DYNAL, INC.
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TEL: 44 23 45 95 FAX: 44 23 16 14

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P.O. Box 204, Carlton South, Victoria 3053
TEL: 1 800 623 453 FAX: (03) 663 6660

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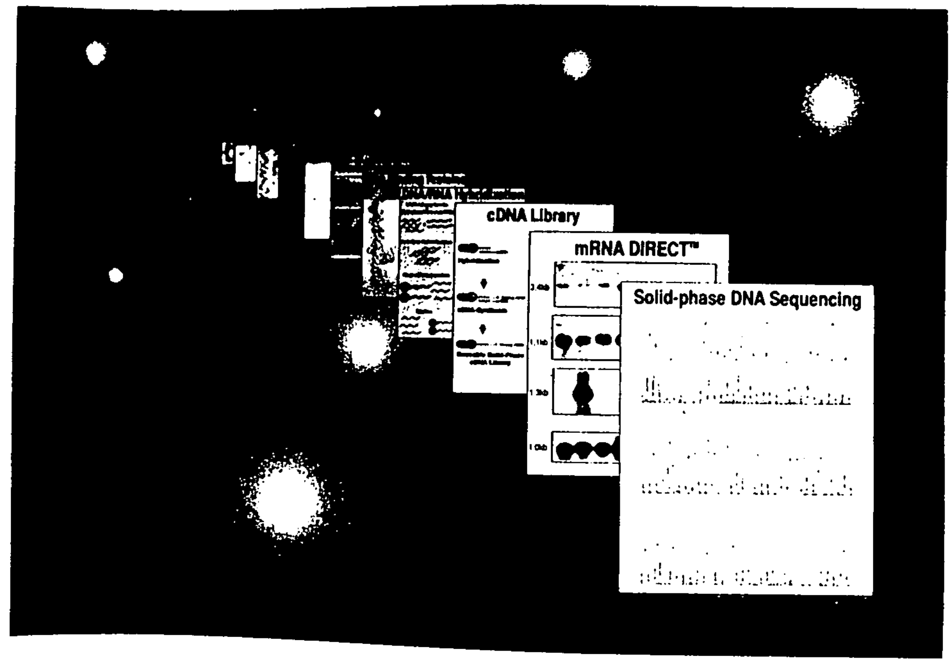

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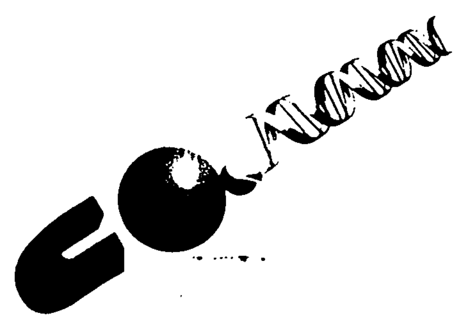


DYNAL®

12.5 CAPTURE OF LARGE INSERTS AND RESTRICTION DIGESTS ON DYNABEADS

12.5.1 General introduction

Genomic libraries in Lambda vectors can be easily constructed, stored and handled, but a major drawback is the difficulty to separate the vector sequence from the insert. To be able to perform shotgun sequencing or genomic walking, the Lambda arms have to be separated from the insert.

12.5.2 Principle of the method

One method to overcome this problem is described by Elgar and Brenner (1). They make use of the cohesive ends of Lambda to biotinylate the DNA by a fill-in reaction using Klenow polymerase in combination with one or two biotin-nucleotides. Any annealed cohesive ends are melted before the fill in reaction of the overhangs. After the incorporation, the DNA is bound to Dynabeads M-280 Streptavidin. The bound Lambda phage DNA is collected with a Dynal MPC, washed, resuspended in an appropriate restriction enzyme buffer and the DNA is cleaved using an enzyme that cuts in the phage polylinker but nowhere else in the vector arms, see Figure 12.5. The beads with the attached vector arms are separated from the insert by magnetic capture.
The released DNA can be used directly for shotgunning after shearing or digestion with a frequent cutter.

Sanford and Elgar (2) describe a modification of this method. The cohesive ends are biotinylated as described above, but the DNA is cut with an enzyme which has no sites in the vector arms or the polylinker. This liberates internal fragments of the insert and leaves the end fragments attached to the arms. The Lambda arms with the end fragments are captured on Dynabeads M-280 Streptavidin, washed and then the end fragments are released by digestion with a restriction enzyme cutting the polylinker. These fragments can be cloned into a plasmid vector or be radiolabeled after separation by agarose gel electrophoresis, see Figure 12.5.
This method allows directional walking from a Lambda clone without first having to obtain a detailed restriction map.

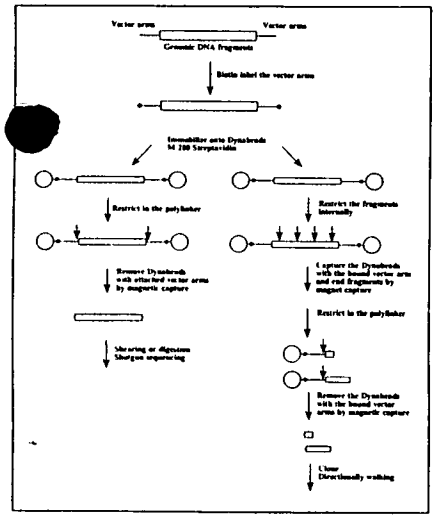


Figure 12.5 A schematic illustration of the principle of capture of large inserts and digests on Dynabeads. Note that in many cases one fragment can bind both ends to the same bead.

12.5.3 References

1. Elgar G.S, Brenner S
   A novel method for isolation of large insert DNA from recombinant lambda DNA.
   Nucleic Acids Research 1992;20:4667
   (Ref. Number: 835)
2. Sanford R.N, Elgar G.S
   A novel method for rapid genomic walking using lambda vectors.
   Nucleic Acids Research 1992,20:4665-4666
   (Ref. Number: 834)

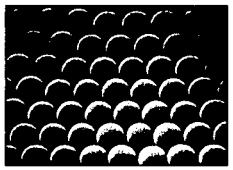
A. DYNABEADS M-280 - PHYSICAL CHARACTERISTICS

Dynabeads M-280 are uniform, monodisperse, superparamagnetic, polystyrene beads.

Diameter: 2.8 μm +/- 0.2 μm (C:V max 3%)
Surface area: 5-8 m^2/g
Specific gravity: approx. 1.3 g/cm^3
Iron content: approx. 13%
Magnetic mass susceptibility: 100 +/- 25x10^{-6} m^3/kg

THE COMPANY

Dynal was formed in 1986 as a joint venture between Dyno Industrier A.S, one of Norway's major industrial companies and Apothekernes Laboratorium A.S, a renowned pharmaceutical company. The company was founded to develop product and applications based on patented superparamagnetic particles, today known as Dynabeads®.



Dynal is based in Oslo, Norway and includes all Research & Development, Production and Quality Control facilities. In addition, Dynal has developed an extensive international sales and marketing organization consisting of subsidiaries, limited partnerships and distributors.

This international network provides Dynal with the flexibility to meet the various demands of a diverse market. Through its business units, Dynal is able to provide highly trained representatives to answer technical questions, arrange product seminars and maintain quality communication with our customers.

The Molecular Biology Division at Dynal continues to grow rapidly since it's introduction in 1989. By maintaining close contact with the needs of our customers we have been able to create new products as well as further develop and continually improve our existing products. The second edition of the Molecular Biology Technical Handbook contains protocols, technical tips and references to innovative Dynabeads applications. These protocols are now in use worldwide by *well known* researchers and scientists, and a comprehensive literature service is available upon request.

The focus of the Dynal organization is to provide the highest quality products and unsurpassed customer service. Our organization is dedicated to our customers' needs. We believe product quality and performance along with technical support are the keys to customer loyalty. We realize that our future depends upon your success.

Dynal is grateful of the contributions and comments which have gone into making this a comprehensive yet accessible document designed for use both at the laboratory bench and in experimental design.

DYNAL LITERATURE SERVICE

More than 350 publications describing different molecular biology applications where Dynabeads have been used, are documented in our Literature Service. Totally, over 1300 publications describing cellular immunology, molecular biology and microbiology applications using Dynabeads are collected in the Dynal Literature Service.

DYNAL LITERATURE AWARD

We will award NOK 10.000 as a travel grant to the first author of at least one paper published in a year. The winner is selected among papers published in a calender year, and known to Dynal within April 1st the following year.
The selection is performed by a scientific committee named by Dynal.

Table of Applications

Table of Contents

1. SOLID-PHASE DNA SEQUENCING

1.1 General introduction

Dynabeads® M-280 Streptavidin provide a unique solid support for direct sequencing of PCR-amplified cloned and genomic DNA (1). This solid-phase approach provides reproducible DNA sequencing results with high yields. The use of Dynabeads M-280 Streptavidin to immobilize the DNA template eliminates time-consuming subcloning, ethanol precipitation, phenol extraction and centrifugation steps and improves the quality of the electrophoretic resolution. Dynabeads M-280 Streptavidin enable efficient production of single-stranded DNA with simultaneous removal of PCR buffers, dNTPs and PCR primers. The immobilization of the PCR product also makes it possible to avoid competition between the sequencing primer and the complementary strand of the template that occurs if double-stranded DNA fragments are used directly. The presence of the Dynabeads in the sequencing reaction does not inhibit the enzymatic activity of the sequencing enzyme. In this chapter, protocols and reference applications for solid-phase DNA sequencing of PCR products are described.

1.2 Methodology

The solid-phase DNA sequencing scheme first described by Hultman *et al.* (1, 2) is outlined in Figure 1.1. First, biotin is introduced into one of the strands of the DNA during the PCR amplification using one of the primers biotinylated in the 5' end. The PCR product is then immobilized through the extremely stable interaction between biotin and streptavidin (K_d=10^{-15}M) onto Dynabeads with covalently coupled streptavidin on the surface. After immobilization, the Dynabeads with the immobilized DNA are washed extensively to remove all the reaction components resulting from the amplification.

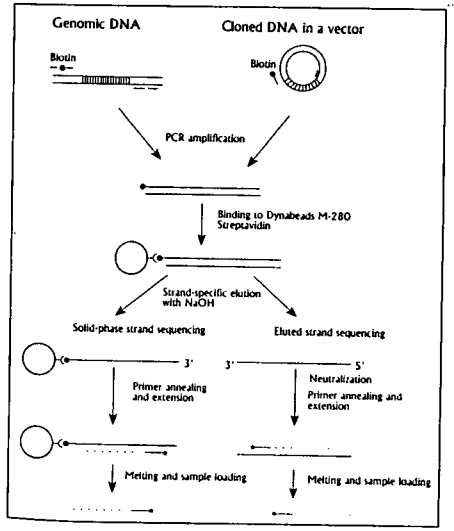


Figure 1.1 Schematic diagram for solid-phase DNA sequencing directly from cloned and genomic DNA.

The immobilized double-stranded DNA is converted to a single-stranded template by incubation with 0.10 M NaOH for 5 minutes and subsequent magnetic separation. Both the immobilized single-stranded template and the eluted strand prepared in this manner are suitable for manual and automated solid-phase DNA sequencing (1, 2) by standard Sanger dideoxy DNA

sequencing (3). A 100% single-stranded template is produced, without interference from primers, free nucleotides or the complementary strand.

After the sequencing reaction, the extended material is purified by magnetic separation, separated from the template strand with formamide and loaded directly onto a sequencing gel.

There are numerous examples in which solid-phase DNA sequencing has proven to be a reliable tool for DNA analysis, see reference applications section 1.7.

1.3 Materials required

To prepare single-stranded DNA for subsequent solid-phase DNA sequencing, the following material/equipment will be required:

- Dynabeads M-280 Streptavidin (10 mg/ml)
- Magnetic Particle Concentrator - Dynal MPC® (see Appendix B).
- Forward and reverse PCR primer (one biotinylated) (see below)
- Binding & Washing Buffer
- Sterile water
- A thermal cycler (DNA Thermal Cycler 480, GeneAmp® PCR System 9600 or GeneAmp® PCR System 2400, Perkin Elmer Corp., Norwalk, CT, USA)
- PCR buffer containing the dNTPs
- *Taq* DNA Polymerase (Perkin Elmer Corp., Norwalk, CT, USA)
- **Fresh** 0.1 M NaOH
- TE buffer
- Neutralizing solution (for DNA sequencing of the eluted strand)
- Toothpicks (for cloned DNA)
- Test tubes, glassware, pipettes
- Tilting/rotation apparatus
- Any DNA sequencing kit for standard Sanger sequencing
- Internal sequencing primers (see later in this section)

See section 1.6 for recipes of current buffers and solutions.

PCR primers:
Each PCR amplification should be performed with a primer set that consists of one biotinylated and one non-biotinylated primer. Dynal recommends biotinylation of oligonucleotide primers at the 5' end. Biotinylation of oligonucleotide primers is described in Appendix E.

For the amplification of **genomic DNA**, specific biotinylated primers for the target gene/sequence must be designed and synthesized.

For the amplification of **cloned DNA**, Dynal recommends to use biotinylated and non-biotinylated versions of the following forward and reverse PCR primers:

forward: 5'-CGCCAGGGTTTTCCCAGTCACGACG-3'
reverse: 5'-GCTTCCGGCTCGTATGTTGTGTGG-3'

These PCR primers are complementary to conserved sequences upstream and downstream of the multiple cloning site (MCS) in common cloning vectors (e.g. pUC, M13 and pBluescript®).

Note: λgt11 is not a suitable vector for use with the recommended forward and reverse PCR primers.

Table 1.1 on the next page indicates the locations of forward and reverse PCR primers as well as common sequencing primer priming sites on current cloning vectors. These vectors contain *E.coli* β-galactosidase sequences used for blue/white colour selection.

Table 1.1 Binding regions at different cloning vectors for recommended PCR primers and sequencing primers.

Cloning Vector	Size	Binding region* for forward PCR primer	Binding region for forward sequencing primer	Multiple cloning sites (MCS)	Binding region for reverse sequencing primer	Binding region for reverse PCR primer	Size of the PCR product without any insert
pUC7 [5,6,7]	2674 bp	352-376	359-375	397-442	469-453	519-496	168 bp
pUC8	2665 bp	352-376	359-375	397-433	460-444	510-487	159 bp
pUC9	2665 bp	352-376	359-375	397-433	460-444	510-487	159 bp
pUC12	2680 bp	352-376	359-375	397-448	475-459	525-502	174 bp
pUC13	2680 bp	352-376	359-375	397-448	475-459	525-502	174 bp
pUC18	2686 bp	352-376	359-375	397-454	481-465	531-508	180 bp
pUC19	2686 bp	352-376	359-375	397-454	481-465	531-508	180 bp
pUC118	3.2 kb	352-376	359-375	397-454	481-465	531-508	180 bp
pUC119	3.2 kb	352-376	359-375	397-454	481-465	531-508	180 bp
M13mp7 [5,6,7,8]	7237 bp	6321-6297	6314-6298	6231-6276	6204-6220	6154-6177	168 bp
M13mp8	7228 bp	6312-6288	6305-6289	6231-6267	6204-6220	6154-6177	159 bp
M13mp9	7228 bp	6312-6288	6305-6289	6231-6267	6204-6220	6154-6177	159 bp
M13mp10	7243 bp	6327-6303	6320-6304	6231-6282	6204-6220	6154-6177	174 bp
M13mp11	7243 bp	6327-6303	6320-6304	6231-6282	6204-6220	6154-6177	174 bp
M13mp18	7249 bp	6333-6309	6326-6310	6231-6288	6204-6220	6154-6177	180 bp
M13mp19	7249 bp	6333-6309	6326-6310	6231-6288	6204-6220	6154-6177	180 bp
pBluescript® SK (+/-) [6,8,10]	2958 bp	576-600	583-599	657-759	828-812	878-855	303 bp
pBluescript® II SK (+/-)	2961 bp	573-597	580-596	657-759	828-812	878-855	306 bp
pBluescript® II KS (+/-)	2961 bp	573-597	580-596	657-759	828-812	878-855	306 bp
Lambda ZAP® II [6,10]	40.82 kb	same distances as for pBluescript® SK (-)					303 bp
pGEM®-3Z [6,11,12]	2743 bp	2679-2703	2686-2702	5-61	128-112	178-155	243 bp
pGEM®-4Z	2746 bp	2677-2701	2684-2700	7-63	126-110	176-153	243 bp
pGEM®-3Zf(+/-)	3199 bp	3133-3157	3140-3156	5-61	120-104	170-147	237 bp
pGEM®-5Zf(+/-)	3003 bp	2937-2961	2944-2960	10-113	177-161	227-204	294 bp
pGEM®-7Zf(+/-)	3000 bp	2934-2958	2941-2957	10-110	174-158	224-201	291 bp
pGEM®-9Zf(+/-)	2925 bp	2837-2861	2844-2860	5-54	119-103	169-146	258 bp
pGEM®-11Zf(+/-)	3223 bp	3157-3181	3164-3180	19-85	144-128	194-171	261 bp
pGEM®-13Zf(+)	3181 bp	3115-3139	3122-3138	11-44	102-86	152-129	219 bp
pCR™II [13]**	3932 bp	457-433	450-434	269-381	205-221	155-178	303 bp

*) The binding region is given in the 5'-3' direction.
**) The pCR™II is identical to the TA Cloning® Vector designed for direct cloning of PCR products manufactured by Invitrogen Corporation.

Note: Blue/white colour selection: Cloning vectors containing the portion of the *lacZ* gene providing α-complementation can be used. When a cloned insert interrupts the *lacZ* α-peptide in the multiple cloning site (MCS), no complementation occurs and transfected or transformed cell colonies appear white when plated on indicator plates.

Sequencing primers:
For sequencing of **genomic DNA**, specific sequencing primers for the amplified target gene/sequence must be designed and synthesized. The use of internal sequencing primers different from the PCR primers is recommended (see section 1.4.1, step 3a).

There are many commercially produced primers for sequencing of **cloned DNA**. The most common primers available are for the M13mp/pUC vectors, but these primers are also suitable for the other vectors indicated in Table 1.1. In this table the following forward and reverse sequencing primer are used as examples:

Forward Sequencing Primer, M13/pUC: 5'-GTTTTCCCAGTCACGAC-3'
(-40, 17-mer)

Reverse Sequencing Primer, M13/pUC: 5'-CAGGAAACAGCTATGAC-3'
(-29, 17-mer)

If you are performing cycle sequencing, you may also use the non-biotinylated version of the recommended forward and reverse PCR primers as sequencing primers (4).

1.4 Protocols

1.4.1 Template preparation

This protocol is used for preparing immobilized PCR-amplified single-stranded templates for subsequent solid-phase DNA sequencing from cloned and genomic DNA samples.

1. Preparing the Dynabeads M-280 Streptavidin

a. Wash **5 - 20** µl (depending on sequencing chemistry; see Note) of Dynabeads M-280 Streptavidin with **20** µl of 2X Binding and Washing (B&W) buffer each time. See section 1.6 for B&W buffer recipe.
The Dynabeads may be washed in bulk. Simply multiply the volumes written in boldtype with the number of sequencing reactions.

Note: **T7 DNA polymerase (Sequenase®) chemistry**

- use 20 µl (200 µg) of Dynabeads per DNA template.

***Taq* cycle sequencing chemistry**

- use 10 µl (100 µg) of Dynabeads per DNA template > 250 base pairs.
- use 5 µl (50 µg) of Dynabeads per DNA template < 250 base pairs.

b. Resuspend the Dynabeads in 40 µl of 2X B&W buffer.

2. Bacterial lysis (for cloned DNA only)

a. Pick a fresh, single bacterial colony (plaque) of the appropriate *E.coli* host containing the inserted plasmid/phage to be sequenced from an agar plate using a sterilized toothpick.

b. Suspend the colony in 8-10 µl sterile water in a PCR tube.
Transfer 5 µl of this suspension to another PCR tube and run the thermocycler for 5 minutes at 96°C.

3. PCR amplification guidelines

a. Use up to 0.5 µg of **genomic DNA** and 5-10 pmoles of each specific flanking primer (one biotinylated and one non-biotinylated) in the PCR reaction. For **cloned DNA**, use approx. 5-10 ng plasmid/phage DNA in 1-2 µl TE buffer.
It is very important to use only the recommended amount of PCR primer, because an excess of unincorporated biotinylated primer will compete with biotinylated PCR products and decrease the amount of amplified product bound to the bead surface.
The recommended primers for amplification of cloned DNA (see section 1.2) are designed for use at high annealing temperatures. A 25 cycle PCR program with denaturation for 30 seconds at 96°C, annealing for 1 minute at 65°C and extension for 2 minutes at 72°C is recommended. See also point d. below.

b. A 3-fold excess of non-biotinylated primer may be used to help drive complete extension of the biotinylated primer. This will further reduce the possibility that unincorporated biotinylated primer interferes with sequencing (4, 14).

c. It is important to use highly purified biotinylated primers in the PCR amplification (see section E.2.6). This will result in a PCR product which has a high biotin content.

- all biotin reagents should contain a spacer arm, at least a 6-carbon linker, to reduce steric hindrance.
- Dynal recommends using reverse-phase HPLC or FPLC® purified biotinylated primers.

See Appendix E for detailed protocols.

d. Dynal recommends completing the PCR amplification with extension at 72°C for 5 minutes to prevent structure-specific endonuclease activity by *Taq* DNA polymerase that may cleave single-stranded DNA (15) and remove the 5' biotin from the primers.
This step will also increase the amount of full length products by ensuring complete extension of incomplete products from previous cycles.

Note: In certain clinical applications, the target DNA may be present in very low concentrations or in a relatively impure sample (16). Both of these problems may occur simultaneously (17).
In such cases the desired products can be generated with increased specificity by using a nested PCR procedure (a two-step PCR with first an outer primer set and secondly an inner primer set where one of the two primers is biotinylated).

Note: Secondary structures in the target DNA may sometimes hinder the extension by the polymerase. The inclusion of additives, such as dimethyl sulfoxide (DMSO) and glycerol in the reaction buffer may allow amplification of DNAs with complex secondary structures (18). DMSO can be added to the reaction to a final concentration of 5 or 10% without inhibiting the PCR efficiency. However, the melting temperature of the primer will be reduced (19).
Alternatively, to minimize the risk for secondary structures you may increase the annealing temperature in the PCR conditions or perform a nested PCR procedure.

4. Immobilization of the PCR product

a. Add 40 µl of the amplified PCR product to 40 µl of the prewashed Dynabeads.

b. Incubate for 15 minutes at room temperature keeping the Dynabeads suspended by gently tipping the tube or plate.

Note: For DNA fragments >1Kb, incubate for 15-60 minutes at 43°C (20). Alternatively, add 50% more Dynabeads to decrease the effect of steric hindrance when immobilizing long PCR products (> 1Kb).

5. Melting the DNA duplex

a. Place the tube/plate containing the immobilized product in a suitable magnet (Dynal MPC) and remove the supernatant with a pipette.

b. Wash the Dynabeads by adding 40 µl of 2X B&W buffer.

Note: The immobilized product can now be stored at 2-8°C for several weeks.

c. Once again, place the washed, immobilized product in the Dynal MPC and remove the supernatant.

d. If you only wish to work with the solid-phase template, resuspend the Dynabeads in **freshly** prepared **0.1 M NaOH** solution (50 µl).

Note: If you wish to recover the non-biotinylated strand for DNA sequencing, use exactly 8 µl **0.100 M NaOH***. Use either freshly prepared NaOH solution or NaOH that has been aliquoted into suitable volumes and stored frozen for not more than 3 months.

e. Incubate at room temperature for 5 minutes.

***) Use a 1.000 ± 0.005 M volumetric solution of NaOH and dilute this to 0.100 M. Aliquot and store the NaOH at -20° C for not more than 3 months.**

6. Separating the DNA strands

a. Collect the Dynabeads on the side of the tube/well using the Dynal MPC, and transfer the NaOH supernatant to a clean tube/well if you wish to sequence the non-biotinylated single-stranded DNA. See section 1.4.3.

b. Wash the Dynabeads (with the immobilized biotinylated strand) once with 50 µl 0.1 M NaOH, once with 40 µl of 1X B&W buffer, and once with 50 µl TE buffer.

Note: All the resuspensions should be made by gentle pipetting or tapping so that any possible aggregates of Dynabeads ("bead complexes") are suspended.

c. Remove the supernatant and adjust the volume with water, according to the template volume of your sequencing protocol.

1.4.2 Solid-phase strand sequencing

All common sequencing kits for manual and automated dideoxy sequencing procedures can be used. Solid-phase sequencing can be performed with all DNA polymerases suitable for sequencing, such as T7 (Sequenase), Klenow, *Taq*, *Tth* and *Bst*. See section 1.7.1 and 1.7.2 for the choice of correct sequencing enzyme for your application.

a. Add the Dynabeads containing the immobilized single-stranded template to the annealing mixture containing the appropriate sequencing primer. Handle the Dynabeads with the immobilized ssDNA as if it were a conventional DNA template.

Note: When working with Dynabeads M-280 Streptavidin for genomic sequencing, Dynal suggests using internal sequencing primers. Do not employ the same primers in the sequencing reaction as those used for producing the PCR product. Failure to follow this suggestion may increases the risk of generating less optimal sequence data due to PCR artifacts (i.e., "false" run-offs due to truncated PCR products) and high background because of false PCR products.
However, under optimal conditions using nested PCR, the non-biotinylated primer used in the second PCR reaction may also be used as the sequencing primer (21).

b. Continue the sequencing reaction according to your own sequencing protocol (annealing and labeling reactions).

c. After completed extension, remove the supernatant containing the excess sequencing primers and unincorporated nucleotides.
It has been reported that the elimination of glycerol and salts from the products of DNA sequencing reactions increases the quality of the sequence data (i.e. band intensity, resolution, and base calling accuracy) (22).
Important note:
Omit this step after cycle sequencing, because in this case the sequencing products are already in the supernatant

d. Elute the newly synthesized strands (the termination products) by adding a formamide solution (stop solution) and heating to 72-95° C for 5 minutes.

Note: Use **fresh** formamide solution. If an old formamide solution is used, heat to 95° C for 5 min. These harsh conditions will destroy the interaction between streptavidin and biotin and release the DNA templates together with the dideoxy fragments. As a result of this dissociation from the bead surface (23), the DNA template can not be resequenced.

Note: For manual sequencing:

• **Sequenase:**	No rinsing is necessary prior to adding the formamide stop solution and gel loading.
For automated sequencing:	
• **Sequenase Dye Primer:**	Rinse the Dynabeads once in TE buffer to stop the reaction and get rid of excess primers, and resuspend in loading buffer.
• **Sequenase Dye Terminator:**	Rinse the Dynabeads 2-4 times in 0.01 M Tris-HCl pH 8.0, 0.1% Tween®-20 to wash away unincorporated dyes. Resuspend in loading buffer.
• ***Taq* Dye Primer:**	Pool the reactions and ethanol precipitate. Resuspend in loading buffer.
• ***Taq* Dye Terminator:**	As with *Taq* Dye Primer, but the extension products need to be cleaned from excess dye terminators with phenol/chloroform extraction (phenol: water:chloroform (68:18:14), twice) or by spin column separation (24).

e. Collect the Dynabeads on the wall of the tube with the Dynal MPC and remove the supernatant containing the dideoxy fragments.

Note: This step may be omitted. The extension sequencing mixture and Dynabeads in formamide solution can be loaded directly onto the sequencing gel without distorting the migration of dideoxy fragments.

f. Load directly each reaction onto the sequencing gel.

Note: **For cycle sequencing,** the immobilized DNA template may be resequenced 2-6 times with only a slight loss of signal at each additional round (4). See section 1.5.4 for further details.

1.4.3 Eluted strand sequencing

The non-biotinylated strand removed during the alkali denaturation step may also be recovered for DNA sequencing. Several successful methods have been reported for the preparation of the eluted, non-biotinylated ssDNA remaining after solid-phase DNA sequencing (2, 25-28). In one of these publications the immobilized strand was sequenced by a Sequenase-based method and the eluted, free strand was successfully sequenced by *Taq* cycle sequencing (28).

In some cases it is difficult to sequence the eluted strand because of problems in adjusting either the pH or the ionic strength in the neutralization step. A method for concentrating the eluted non-biotinylated strand by magnetic separation has recently been developed. This is achieved by labeling the non-biotinylated PCR primer with digoxigenin (Boehringer Mannheim GmbH, Mannheim, Germany) and then using Dynabeads coated with anti-digoxigenin antibodies to capture the eluted strand, after alkali treatment (29).

To recover and determine the nucleotide sequence of the complementary DNA template in the alkali supernatant, all the following **five** procedures have given successful results - you may choose the one best suited to your application:

Methods:

1. Neutralize the alkali supernatant with precisely 4 µl **0.200 M HCl*** and 1 µl 1.0 M Tris-HCl (pH adjusted to optimum of sequencing enzyme) (2).

Mix immediately with a pipette and adjust the the volume with water according to your sequencing protocol.

Note: Always use the same pipette for both NaOH and HCl, as small differences in calibration between different pipettes can cause neutralization problems.

***) Use a 1.000 ± 0.005 M volumetric solution of HCl and dilute this to 0.200 M. Aliquot and store the HCl at -20°C for not more than 3 months.**

2. Elute the non-biotinylated ssDNA from pelleted beads by the addition of 300 µl 0.15 M NaOH for 5 minutes. After magnetic separation, transfer the supernatant to a new tube. Precipitate by neutralizing with the addition of 150 µl 5 M ammonium acetate pH 6.6, followed by isopropanol precipitation using 2 µg of nucleic acid free glycogen as a co-precipitant (25).
3. Recover the unbiotinylated strands by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol to the NaOH washes. Incubate at -20°C for 2 hours. Centrifuge the samples at 12,000 g for 10 minutes and wash the pellets twice with 70% ethanol, vacuum-dry and redissolve in 5 µl of water (26).
4. An alternative method by Lee and Vacquier (27) circumvents the need for accurate neutralization and precipitation of the alkali supernatant by using a Centricon®-30 microconcentrator. In this way, accurate neutralization of the NaOH following separation of the non-biotinylated strand is not required.
5. In the kit manual for the PRISM™ Solid Phase Sequenase® Dye Terminator DNA Sequencing Kit from Perkin Elmer - Applied Biosystems Division another method for recovering the non-biotinylated strand for sequencing is described (14). This method gives a relatively low DNA recovery due to loss during precipitation. Because of this, a cycle sequencing (dye terminator or dye primer) protocol is recommended rather than a Sequenase-based protocol.
   a. Collect the first 100 µl of the main eluate and neutralize with 100 µl of 3 M sodium acetate, pH 5.5.
   b. Precipitate the DNA by adding 200 µl of isopropanol and mix well by vortex mixing.
   c. Incubate at room temperature for 10-20 minutes.
   d. Spin in a microcentrifuge for 15 minutes at 10,000 x g.

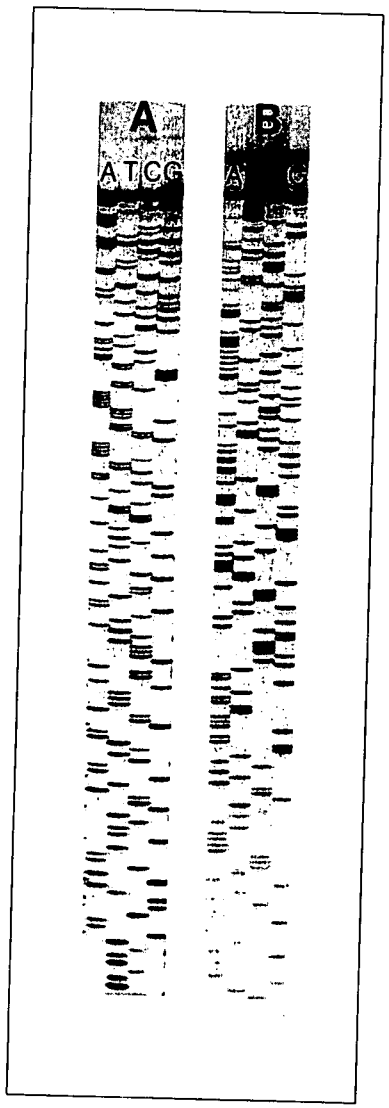


Figure 1.2
Autoradiograms of sequencing gels of the solid-phase strand (A) and also the eluted, complementary DNA strand (B) (27).

   e. Gently remove the supernatant and blot the outside rim of the tube dry.
   f. Briefly microcentrifuge a second time and pipette off the remaining supernatant, being careful not to disturb the DNA pellet.
   g. Wash the pellet with 300 µl of 70% ethanol, spin in a microcentrifuge for 15 minutes.
   h. Repeat step 5 and 6.
   i. Dry in vacuum centrifuge for 2-5 minutes and resuspend the pellet in 5 µl TE.
   j. Place in a freezer or at -20°C until ready for sequencing.
   k. Use the template with any of the sequencing chemistries.

1.5 Technical tips and troubleshooting

Solid-phase DNA sequencing with Dynabeads is the most reliable method for directly sequencing PCR products and usually gives beautiful clear banding patterns on your autoradiograph or fluorogram. However, you may sometimes obtain unsatisfactory results. If this happens, it is important to evaluate your result and use its appearance to deduce the most likely cause of the problems. T.A. Brown has recently published a book which draws on his own experience in attempting to understand what he was doing when he first sequenced DNA (30). This book also includes a troubleshooting guide for manual sequencing which may be of help to you.

In general, each series of sequencing reactions should include one control reaction using high quality DNA. For single-stranded DNA, the use of 1 µg commercially available M13mp18 DNA is recommended.

It is important to remember that problems arising during the PCR amplification may also affect your sequencing data. The most common effect is related to PCR artifacts during the PCR amplification due to secondary structures (hairpin loops). This problem can be overcome by performing a hot start PCR, adding DMSO (5% or 10% final conc.) to the PCR reaction mixture or increasing the annealing temperature in the PCR by 2-3°C.

1.5.1 Sequencing of GC-rich regions

The solid-phase approach is also the method of choice for sequencing of regions with high GC content (i.e. promotor sequences). Template preparation with Dynabeads M-280 Streptavidin permits sequencing of DNA containing GC-rich regions (typically 70-75%) with T7 DNA polymerase without the need for special conditions (except for the use of 7-deaza-dGTP instead of dGTP in the sequencing mixture (2). This has been documented by fluorescence sequencing of a cloned *Streptomyces curacoi* gene.

Sequence analysis of a polymorphic region of the human dopamine D_4 receptor gene has identified 19 unique, highly related 48 base pair repeat sequences using Dynabeads for the template preparation (31). This polymorphic region has GC contents in the range from 75 up to 87%. In this case, 10% DMSO has been used in the reaction mixtures for both the PCR amplification and the sequencing reaction. For one of the two sequencing kits used, the dGTP was replaced by 7-deaza-dGTP. Standard dideoxy sequencing using double-stranded DNA templates of this region was not feasible as the high GC content leads to unreliable sequence data (31).

1.5.2 Handling of secondary structures

Direct sequencing of PCR products by T7 DNA polymerase is prone to problems in GC-rich regions, due to the stability of secondary structure. To overcome these difficulties, Zhang *et al.* (32) have examined the effect of adding formamide to the sequencing reaction. Inclusion of

formamide may increase the intensity of specific bands, dissolve several local secondary structures, and eliminate the presence of most bands which cross over all four lanes. Handling of secondary structures is also described in section 1.4.1, step 3 and in section 1.5.1.

1.5.3 Avoiding mis-priming of DNA template

Mis-priming of DNA template is one of the most common problems associated with the dideoxy chain termination method of DNA sequencing (3). It can be a particular problem with GC-rich primers and templates, or with templates containing repeats and unknown sequences. Thomas *et al.* (33) have found that a simple stringency wash can eliminate mis-priming completely when using Dynabeads for the generation of single-stranded DNA templates. The Dynabead/template/sequencing primer complex was washed twice with pre-warmed Sequence reaction buffer, using the Magnetic Particle Concentrator (Dynal MPC) in the water bath to separate the Dynabeads between the washes.

Figure 1.3 shows the results of sequencing part of the human mitochondrial DNA D-loop region with and without a stringent wash of the complex. The stringency wash proved effective both in removing cross-banding due to mis-priming and in increasing the signal generated from correctly primed template DNA. Furthermore, this improvement occured over a broad temperature range (45 to 55°C) and with a minimum number of washes (two). This method can only be performed on an immobilized single-stranded DNA template which can be separated from the washes.

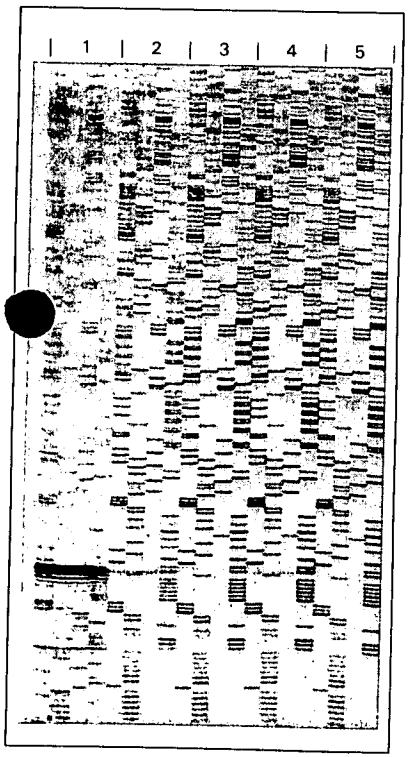


Figure 1.3 Autoradiagram of sequencing gel showing five sets of sequencing reactions, each containing identical template DNA and sequencing primer combinations, but different in their treatment after sequencing primer annealing (33). Sequencing reactions were carried out on sample 1 with no further processing. Sample 2 and 3 were washed once and twice respectively at 45°C and sample 4 and 5 washed once and twice respectively at 55°C. An internal sequencing primer was used.

1.5.4 Reuse of Dynabeads M-280 Streptavidin for cycle sequencing

Once a biotinylated target is immobilized onto Dynabeads M-280 Streptavidin, it is not possible to remove the initial target and add a new biotinylated target in its place. All methods for removal of a biotinylated target from Dynabeads M-280 Streptavidin (see Appendix G) will leave the Dynabeads in an inactive form for binding of biotin (either because the streptavidin binding sites remain blocked or the streptavidin protein is irreversibly denatured and destroyed. For some applications, a biotinylated probe attached to Dynabeads M-280 Streptavidin can be reused to recover another target population. **For cycle sequencing**, the immobilized DNA template may be resequenced 2-6 times with only a slight loss of signal at each additional round (4). In this case the elution step with formamide and heat (see section 1.4.2, step d) is omitted. After completion of the sequencing reaction, decant and process the supernatant (containing the sequencing products) for analysis according to the instructions in the protocol being used. Wash the immobilized template twice in 0.10 M NaOH and twice in TT buffer. Store the immobilized template at 4°C.

1.5.5. Control reaction for template preparation of PCR products

A positive control for use in troubleshooting for template preparation of PCR products from cloned DNA and solid-phase DNA sequencing is available from Dynal upon request. This control can be used with the M13 universal sequencing primer.

1.6 Buffers and solutions

Binding & Washing buffer	A buffer with a final salt concentration of at least 1.0 M is recommended for optimal binding of the DNA template. A suggested buffer follows: Binding & Washing buffer (2X concentration) 10 mM Tris-HCl pH 7.5 1.0 mM EDTA 2.0 M NaCl **Note:** A binding buffer with 3 M LiCl instead of 1 M NaCl has been reported to give an improved binding of large DNA fragments (> 1 Kb) (20). NaCl is substituted by LiCl because NaCl tends to precipitate at high concentrations (LiCl has a higher solubility than NaCl). **See section F.3 and F.4 for optimal binding conditions for DNA fragments of different sizes.**
PCR buffer	20 mM Tris-HCl, pH 8.3 at 20°C 50 mM KCl 0.1% Tween® 20 2.0 mM $MgCl_2$ 200 µM of each dNTP
TE buffer	10 mM Tris-HCl, pH 7.5 1 mM EDTA
Melting solution	0.100 M NaOH
Neutralizing solution	0.200 M HCl and 1M Tris-HCl
TT buffer (for resequencing templates)	10 mM Tris-HCl, pH 8.0 0.1% Tween-20

1.7 Reference applications

1.7.1 Sequenase®-based sequencing

In clinical applications, the Sequenase (T7 DNA polymerase) is the enzyme of choice, because of better peak uniformity compared to other polymerases. Peak uniformity is important for accurate base-calling, especially for the detection of heterozygosity in genomic material (34). In a comparison study between four different sequencing strategies performed by researchers at the Forensic Science Service in the U.K. (20), it was concluded that the most optimal results were obtained on Dynabeads followed by solid-phase DNA sequencing with Sequenase. This sequencing strategy proved to be the most reliable, the most sensitive and the most consistent of the methods attempted.

T7 DNA polymerase (Sequenase)

- High rate of polymerization
- Uniform signal intensity (i.e., detecting mutations and heterozygosity)
- Longer read length

1.7.2 Cycle sequencing with *Taq*® DNA polymerase

The thermostable enzymes poorly incorporate dideoxynucleotides in a non-consistent pattern in comparison with native deoxynucleotides (34). Despite the disadvantages of non-uniform signal intensities, the convenience of rapid sequence generation by cycling the temperature conditions has made *Taq* DNA polymerase useful for sequencing efforts involving cloned DNA (4).

***Taq* DNA Polymerase**

- Higher temperatures reduce secondary structures (i.e., GC rich regions)
- Less DNA template required
- Less Dynabeads M-280 Streptavidin required

1.7.3 Clinical DNA sequencing

Unpredictable mutations in somatic cells are responsible for the large majority of genetic diseases. Genes involved in cell proliferation are often targets for the acquired mutations, which have been found to occur in regions of the DNA sequence that disturb either protein function or transcriptional and/or translational control.

Direct sequencing of PCR-amplified material is the technology of choice for the emerging field of clinical DNA sequencing in the investigation and research of genetic diseases. The nucleotide sequence of infecting bacteria or viruses can also be determined directly from patient samples.

Sequencing with Dynabeads defines precisely the location and nature of mutational changes and represents the ideal mutation scanning technique. An example of direct clinical DNA sequencing is shown in Figure 1.4. It illustrates a point mutation appearing in the p53 gene encoding a nuclear phosphoprotein which is among the most frequent genetic alterations in human solid tumours. The sequencing data easily detects a C →T transition in tumour DNA in a skin cancer study (35).

Table 1.2 on the next page shows the documentation on clinical DNA sequencing where Dynabeads have successfully been used. A number of these publications demonstrate that single point mutations may be easily detected.

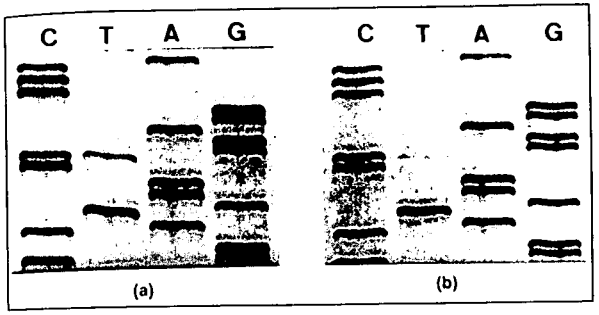


Figure 1.4
(a) Tumour DNA, showing C → T transition at codon 248 (*).
(b) Normal sequence at codon 248 (*) (35).

Table 1.2 A summary of genetic diseases and bacterial/viral infections in alphabetic order where clinical DNA sequencing has been reported.

Genetic disease/Virus	Target gene	Nature of mutation	Authors	Ref.
Acute Lymphoblastic Leukemia (ALL)	FRS gene	DNA deletions, DNA rearrangements	Nizet, Y. *et al.*	36
Alzheimer's disease	APP gene	point mutations	Adroer, R. *et al.*	37
Alzheimer's disease	APP gene	point mutations	Hendriks, L. *et al.*	38
Alzheimer's disease	APP gene	point mutations	Johnston, J. *et al.*	39
Alzheimer's disease	APP gene	point mutations	Mullan, M. *et al.*	40
Aniridia (AN)	PAX6 gene	point mutations, DNA insertions	Davis, A. *et al.*	41
Basal cell carcinoma (BCC)	p53 gene	point mutations	Hedrum, A. *et al.*	42
Bowen's disease	p53 gene	point mutations	Campbell, C. *et al.*	35
C8β deficiency, human	C8B gene	point mutations, sequence comparison	Kaufmann, T. *et al.*	43
Cancer	mouse p53 gene	sequence determination	Jerry, D.J. *et al.*	44
Cancer	rat p53 gene	sequence comparisons	Hulla, J.E. *et al.*	45
Cancer	p53 gene	point mutations	MacGeoch, C. *et al.*	46
Cancer, breast and ovarian	BRCA1 gene	point mutations, sequence determination	Campbell, I.G. *et al.*	47
Cancer, breast	p53 gene	point mutations	Maurer, J. *et al.*	48
Cancer, breast	p53 gene	point mutations, DNA deletions	Thorlacius, S. *et al.*	49
Cancer, lung	CYP2D6 gene	point mutations, DNA deletions	Saxena, R. *et al.*	50
Charcot-Marie-Tooth disease	myelin Po gene	point mutations	Hayasaka, K. *et al.*	51
Charcot-Marie-Tooth disease	myelin Po gene	point mutations	Hayasaka, K. *et al.*	52
Charcot-Marie-Tooth disease (CMTX1)	connexin 32 gene	point mutations, DNA deletions	Fairweather, N. *et al.*	53
Charcot-Marie-Tooth disease	PMP22 gene	point mutations	Nelis, E. *et al.*	54
Charcot-Marie-Tooth disease (CMT)	PMP22 gene	point mutations, DNA rearrangements	Pentao, L. *et al.*	55
Charcot-Marie-Tooth disease	PMP22 gene	point mutations, DNA deletions	Roa, B.B. *et al.*	56
Charcot-Marie-Tooth disease	PMP22 gene	point mutations	Roa, B.B. *et al.*	57
Chlamydia trachomatis	CrP gene	point mutations	Hedrum, A. *et al.*	58
Chlamydia trachomatis	CrP gene	point mutations	Wahlberg, J. *et al.*	59
Cholera	ctxB gene	point mutations	Olsvik, Ø. *et al.*	60

Genetic disease/Virus	Target gene	Nature of mutation	Authors	Ref.
Chronic inflammatory arthritis	TcR $V_\delta 1$ gene	sequence diversity	Bucht, A. *et al.*	61
Chronic Myeloid Leukemial (CML)	fused bcr-abl gene	translocation	Debuire, B. *et al.*	62
Chronic myelogenous leukemia (CML)	p53 gene, N-ras gene	point mutations, translocations, review	Mizutani, S. *et al.*	63
Creutzfeldt-Jakob Disease (CJD)	PrP gene	DNA deletions	Palmer, M.S. *et al.*	64
Cystic fibrosis (CF)	CFTR gene	DNA deletions	Chu, C.-S. *et al.*	65
Cystic fibrosis (CF)	CFTR gene	DNA deletions	Chu, C.-S. *et al.*	66
Cystic fibrosis (CF)	CFTR gene	DNA deletions	Chu, C.-S. *et al.*	67
Cystic fibrosis (CF)	CFTR gene	point mutations	Glavac, D. *et al.*	68
Cystic fibrosis (CF)	CFTR gene	point mutations	Greil, I. *et al.*	69
Cystic fibrosis (CF)	CFTR gene	point mutations, DNA deletions	Hermans, C.J. *et al.*	70
Cystic fibrosis (CF)	CFTR gene	point mutations	Schaedel, C. *et al.*	71
Cystic fibrosis (CF)	CFTR gene	DNA insertions	Will, K. *et al.*	72
Dejerine-Sottas syndrome	myelin Po gene	point mutations	Hayasaka, K. *et al.*	73
Dejerine-Sottas syndrome	myelin PMP22 gene	point mutations	Roa, B.B. *et al.*	74
Denys-Drash syndrome (DDS)	WT1 gene	point mutations, DNA insertions	Baird, P.N. *et al.*	75
Diabetes (NIDDM)	human IRS-1 gene	point mutations	Almind, K. *et al.*	76
Diabetes	mouse IL-2	point mutations, DNA insertions	Ghosh, S. *et al.*	77
Diabetes	mouse Fcgr1 gene	DNA deletions	Prins, J.-B. *et al.*	78
Epidermolysis bullosa simplex (EBS)	K1 and K10 genes	point mutations	Cheng, J. *et al.*	79
Epidermolytic hyperkeratosis (EH)	K1 and K10 genes	point mutations	Cheng, J. *et al.*	79
Epidermol. palmoplantar keratod. (EPPK)	keratin 9 gene	point mutations	Reis, A. *et al.*	80
Epstein-Barr Virus (EBV)	EBNA4 epitope	point mutations	De Campos-Lima, P.-O. *et al.*	81
Familial adenomatous polyposis (FAP)	APC gene	point mutations, DNA deletions	Gebert, J.F. *et al.*	82
Familial adenomatous polyposis (FAP)	APC gene	point mut., DNA delet., DNA insert.	Hamzehloei, T. *et al.*	83
Familial adenomatous polyposis (FAP)	APC gene	point mut., DNA delet., DNA insert.	Mandl, M. *et al.*	84
Familial defective apolipoprot. B (FDB)	apoB gene	point mutations	Leren, T.B. *et al.*	34
Familial glucocorticoid deficiency (FGD)	ACTH receptor gene	point mutations	Weber, A. *et al.*	85
G6PD deficiency	G6PD gene	point mutations	Rovira, A. *et al.*	86
Gastric carcinoma	p53 gene	point mutations	Seruca, R. *et al.*	87
Genetic disorders	human apolipoprot. E gene	point mutations	Syvänen, A.-C. *et al.*	88
Hereditary coproporphyria (HC)	CPX gene	point mutations	Delfau-Larue, M.-H. *et al.*	89
Hereditary coproporphyria (HC)	CPX gene	point mutations	Martasek, P. *et al.*	90
HIV-1	RT gene	sequence comparison	Albert, J. *et al.*	91
HIV-1	RT gene	point mutations, sequence comparison	Cox, S.W. *et al.*	92
HIV-1	pol gene	sequence comparison	Albert, J. *et al.*	93
HIV-1	pol gene	point mutations, sequence comparison	Wahlberg, J. *et al.*	94
HIV-1	env gene and nef gene	sequence diversity	Andersson, B. *et al.*	95

Genetic disease/Virus	Target gene	Nature of mutation	Authors	Ref.
HIV-1	RT gene	point mutations	Larder, B.A. *et al.*	96
HIV-1	V3 loop	sequence comparison	Leitner, T. *et al.*	97
HIV-1	env gene	sequence determination	Rolfs, A. *et al.*	28
HIV-1	V3 loop	sequence comparison	Scarlatti, G. *et al.*	98
HIV-1	V3 loop	point mutations, sequence comparison	Wahlberg, J. *et al.*	99
Hodgkin disease (HD)	p53 gene	point mutations	Gupta, R.K. *et al.*	100
Human cytomegalovirus (HCMV)	IE-1 gene	point mutations	Brytting, M. *et al.*	101
Human diseases	mutation detection	review article	Cotton, R.G.H. *et al.*	102
Human hepatocellular carcinoma	p53 gene	point mutations, DNA deletions	Challen, C. *et al.*	103
Hyperfunctioning thyroid adenoma	thyrotropin receptor gene	point mutations	Parma, J. *et al.*	104
Infectious diseases		review article	Olsvik, Ø. *et al.*	105
Lesch-Nyhan (LN) syndrome	HPRT gene	point mutations, DNA deletions	Gibbs, R.A. *et al.*	106
Lobar Atrophies (LA)	PrP gene		Owen, F. *et al.*	107
Medullary thyroid carcinoma (MTC)	RET gene	point mutations	Zedenius, J. *et al.*	108
Meningioma	NF2 gene	point mutations, DNA deletions	Ruttledge, M.H. *et al.*	109
Mutation detection		review article	Grompe, M.	110
Neurofibromatosis	NF1 gene	DNA insertions	Bleyl, S. *et al.*	111
Neuropsychiatric disorders	DRD4 gene	sequence comparison, heterozygosity	Lichter, J.B. *et al.*	31
Plasmodium falciparum	Pf155 region/RESA	DNA detection	Lundeberg, J. *et al.*	112
Plasmodium falciparum	Pf155 gene	sequence determination	Wahlberg, J. *et al.*	113
Plasmodium falciparum	PfDHFR/TS gene	point mutations	Holmberg, M. *et al.*	114
Porcine malignant hyperthermia	CRC cDNA fragment	point mutations	Harbitz, I. *et al.*	115
Phenylketonuria (PKU)	PAH gene	point mutations	Eiken, H.G. *et al.*	116
Primary amenorrhoea	FSH-β subunit gene	DNA deletions	Matthews, C.H. *et al.*	117
Pure gonadal dysgenesis	SRY gene	point mutations	Affara, N.A. *et al.*	118
Saccharomyces cerevisiae	Cox1 gene	DNA insertions	Mueller, M.W. *et al.*	119
Schwannomas	NF2 gene	point mut., DNA insert., DNA delet.	Twist, E.C. *et al.*	120
Sex determination, monkey	SRY gene	point mutations	Whitfield, L.S. *et al.*	121
X-linked agammaglobulinemia (XLA)	btk gene	point mutations, DNA insertions	Bradley, L.A.D. *et al.*	122
X-linked disease (XSCID)	IL-2Rγ gene	point mutations	Noguchi, M. *et al.*	123
XY sex reversal	SRY gene	point mutations	Iida, T. *et al.*	124

1.7.4 Sequence-based HLA typing

It has become widely appreciated during the past two decades that the HLA system plays a central role in controlling immune responses and influencing susceptibility to a large number of diseases.

During the last few years there has been spectacular progress in the development of PCR-based HLA typing methods, which offer substantial improvements compared to genomic DNA restriction fragment length polymorphism (RFLP). Both hybridization with sequence-specific oligonucleotides (PCR-SSO) and amplification with sequence-specific primers (PCR-SSP) have already found great favour in the HLA-typing area (125).

Sequence-based typing (PCR-SBT) is playing an increasingly important role in research on tissue typing for transplantation (126). Direct solid-phase DNA sequencing in particular, with its reliability and capacity for revealing the entire nucleotide sequence coding for any HLA antigen and detecting variations as slight as a single base change, offer the potential of providing a comprehensive solution to the tissue-typing problem (126). Furthermore, this technique is amenable to automation, making it potentially ideal for routine clinical use (126). Kaneoka *et al.* (127,128) have established a method for typing HLA-DR genes by direct sequencing of PCR amplified DNA using Dynabeads as the solid support. Spurkland *et al.* (129) have also established a similar method for HLA-DR and HLA-DQ genes. Sequencing ladders obtained by these procedures are easily readable, the patterns can be interpreted in HLA homozygous as well as heterozygous individuals, and sequence differences or similarities between the Bone Marrow Transplantation (BMT) donor and recipient can be directly identified (129).

PCR-SBTof HLA-DPB alleles using Dynabeads for the template preparation has been compared to PCR-SSO and the results correlate perfectly with the PCR-SSO-typing results (130).

In certain clinical sequence-based analysis (i.e. mutation screening), PCR amplification and direct DNA sequencing of a specific HLA locus with high degree of polymorphisms (i.e. HLA-DQB1) in parallel, allows a genetic "fingerprint" for each patient, and makes it easier to avoid sample mix up (42).

Both manual and automated sequencing techniques using a variety of sequencing strategies have been used for sequence-based typing. A summary of the different HLA loci, which have been determined by sequence-based typing using Dynabeads as the solid support, is presented in Table 1.3.

Table 1.3 Sequence-based typing of different HLA locus using Dynabeads as the solid support and choice of sequencing strategy.
ABI373A is the sequencer from Perkin Elmer - Applied Biosystems Division, Foster City, CA, USA.
ALF is the sequencer from Pharmacia Biotech AB, Uppsala, Sweden.
DNA4000 is the infrared sequencer from LI-COR, Inc., Lincoln, NE, USA.

HLA locus	Sequencing strategy	Authors	Ref.
HLA-DPB1	Automated sequencing (*Taq* Dye Primers) - ABI373A	Tilanus, M.	126
HLA-DPB1	Automated sequencing (*Taq* Dye Primers) - ABI373A	Versluis, L.F. *et al.*	130
HLA-DPB1	Automated sequencing (T7 Dye Primers) - DNA4000	Knipper, A.J. *et al.*	131
HLA-DQB1	Manual sequencing (^{35}S)	Spurkland, A. *et al.*	129
HLA-DQB1	Automated sequencing (T7 Dye Primers) - ALF	Hedrum, A. *et al.*	42
HLA-DQB1	Automated sequencing (T7 Dye Primers) - ALF	Hedrum, A. *et al.*	132
HLA-DQB1	Manual sequencing (^{35}S)	Knipper, A.J. *et al.*	131
HLA-DQB1	Automated sequencing (T7 Dye Primers) - DNA4000	Knipper, A.J. *et al.*	131
HLA-DRB1	Manual sequencing (^{32}P)	Kaneoka, H. *et al.*	127
HLA-DRB1	Automated sequencing (*Taq* Dye Terminators) -ABI373A	Kaneoka, H. *et al.*	128
HLA-DRB1	Manual sequencing (^{35}S)	Spurkland, A. *et al.*	129
HLA-DRB1	Automated sequencing (T7 Dye Primers) - DNA4000	Knipper, A.J. *et al.*	131
HLA-DRB3	Automated sequencing (T7 Dye Primers) - DNA4000	Knipper, A.J. *et al.*	131
HLA-DRB4	Automated sequencing (T7 Dye Primers) - DNA4000	Knipper, A.J. *et al.*	131
HLA-DRB5	Automated sequencing (*Taq* Dye Terminators) - ABI373A	Kaneoka, H. *et al.*	128
HLA-DRB5	Automated sequencing (T7 Dye Primers) - DNA4000	Knipper, A.J. *et al.*	131
RING4 gene	Manual sequencing (^{32}P)	Kelly, A. *et al.*	133
RING11 gene	Manual sequencing (^{32}P)	Kelly, A. *et al.*	133
RING11 gene	Manual sequencing (^{32}P)	Powis, S.H. *et al.*	134

1.7.5 Sequence-based bacterial typing

In recent years sequence analysis of ribosomal RNA (rRNA) genes has been very useful for the identification of and for the discrimination between closely related bacterial species (135, 136). In general, rDNA complementary to the rRNA is generated by PCR. rRNA sequences can facilitate species identification using a rRNA reference sequence library available as a computer database, and generic PCR primers from conserved areas of prokaryote rRNA genes can be used for most bacterial species (135). This technology has made it possible to obtain data corresponding to 400–600 bases from 10–20 strains within a single working day (137). A recent study shows that DNA sequencing is a useful epidemiological tool for the identification and characterization of clinically important isolates (135). Such epidemiological information is useful for investigation of sources and routes of transmission.

Sequence analysis of rRNA genes have also applications in evolutionary studies and for diseases associated with unculturable bacterial strains (138). The 16S rRNA sequence mutates at a rate that makes it useful as an evolutionary clock. For example, the evolutionary distance from one organism to another can be calculated from the number of nucleotide differences between their respective 16S rRNA sequences. Because portions of all 16S rRNA genes are highly conserved, these genes can be amplified from uncharacterized organisms with broad-range primers used in the PCR. This method has been used to identify a unique *Bacillus* species, the unculturable organism responsible for Whipple's disease (138). The bacterial 16S rRNA sequence was amplified directly from tissues of patients, and sequenced.

Automated solid-phase DNA sequencing with T7 DNA polymerase can be used for efficient generation of sequence data from rRNA genes (139). Primary data with more even peak heights are obtained by sequencing with T7 DNA polymerase than by other sequencing methods such as cycle sequencing. The results have suggested that the direct solid-phase DNA sequencing procedure with Dynabeads is a powerful tool for identification of mycoplasmas and is also useful in taxonomic studies (139). Furthermore, the method is suitable for many diagnostic applications in bacteriology, and will be particularly useful when more rRNA sequences have been deposited in the data base (139). Bacterial typing based on direct automated solid-phase DNA sequencing has the following advantages (140):

- Reverse transcription is not needed (Depends on whether rRNA or ribosomal DNA (rDNA) is used as starting material. Some may prefer reverse transcription of rRNA as starting material, because of the high copy number per cell.)
- Cloning procedures are not required
- Analysis of non-cultivable species is possible
- The solid-phase procedure is suitable for automation

A universal and reliable sequencing approach suitable for complete automation for evolutionary and phylogenetic studies of most plant species, based on chloroplast DNA analysis, has also been developed (141). In a similar way, sets of nested or hemi-nested primers have been made to amplify the highly conserved chloroplast *trnL* and *trnF* genes. PCR products obtained from these regions in the chloroplast genome from a variety of species have been sequenced. The results have shown that these regions are useful in phylogenetic studies at different taxonomic levels (141).

Sequence-based bacterial typing using Dynabeads as the solid support is summarized in Table 1.4.

Table 1.4 Sequence-based bacterial typing of different bacterial species using Dynabeads as the solid support with belonging target rRNA gene.

Species	Target gene/sequence	Authors	Ref.
Campylobacter	23S rRNA gene	Eyers, M. *et al.*	142
Campylobacter jejuni	23S rRNA gene	Van Camp, G. *et al.*	143
Escherichia coli	16S rRNA gene	Pettersson, B. *et al.*	139
Legionella bozemanae	5S rRNA gene	Rolfs, A. *et al.*	28
Legionella pneumophila	5S rRNA gene	Rolfs, A. *et al.*	28
Magnetotactic bacteria	ssu rRNA gene	DeLong, E.F. *et al.*	144
Mycoplasmas	16S rRNA gene	Pettersson, B. *et al.*	139
Mycoplasmas	16S rRNA gene	Pettersson, B. *et al.*	140

1.7.6 Forensic and population studies

1.7.6.1 Sequencing of mitochondrial DNA

Sequence analysis of mitochondrial DNA (mtDNA) has applications in forensics, population and human diversity studies, genealogical studies, and species identification.
Mitochondrial DNA exists in mitochondria of all animal cell types as a closed, circular, double-stranded molecule. Human mtDNA is 16,569 bp in length (145).

There are several characteristics of mtDNA that make it a useful tool for human identification. The following examples illustrate instances in which mtDNA analysis may be preferred over nuclear DNA (145).

1. **A sample containing limited amounts of DNA, such as a hair shaft.**
   Because mtDNA is present in high copy number (single mammalian cells contain from several hundred to 10,000 mitochondrial genomes, the chances of successful PCR amplification even from very small numbers of cells are good.
2. **Samples that are highly degraded, such as ancient remains, or bone, and teeth.**
   Because mtDNA is circular, it is less susceptible to exonuclease degradation. Because of the high copy number, the chances that an undegraded fragment will survive is greater.
3. **Samples of remains where only distant relatives are known.**
   Because mtDNA is maternally inherited, as long as an individual shares maternal descent with a candidate sample source, he or she can be used to verify identity (48).

Researchers at the Forensic Science Service in U.K. have compared four different amplification and sequencing strategies in order to develop a robust protocol for the automated analysis of mtDNA from semen and hair shaft (20). It was concluded that optimal results were obtained on Dynabeads followed by solid-phase DNA sequencing with Sequenase. This sequencing strategy appeared to be the most reliable, the most sensitive and the most consistent of the methods attempted.

Sullivan *et al.* (146) have continued the development towards a fully automated forensic DNA test where the solid-phase approach has been used for the analysis of amplified hypervariable segments of mtDNA. This approach enabled good quality sequences to be generated from severely degraded and low-concentration DNA samples, such as from human bones buried for over 50 years, thereby providing a definitive identification test.
This method have also been used to sequence mtDNA from 100 unrelated British White Caucasian to investigate the nucleotide diversity (147).

Nine skeletons found in a shallow grave in Ekaterinburg, Russia, in 1991, were tentatively identified by Russian forensic authorities as the remains of the last Tsar, Tsarina, three of their five children, the Royal Physician and three servants.
Sequence analysis of mtDNA with Dynabeads performed by researchers at the U.K. Forensic Science Service mentioned above, reveals an exact sequence match between the putative Tsarina and the three children with a living maternal relative (148). The authors of this paper have concluded that the DNA evidence supports the hypothesis that the remains are those of the Romanov family.

Solid-phase sequencing has been used in a population study to examine nucleotide sequence variation in the mtDNA from 90 humpback whales collected from the three major oceanic basins (149).

1.7.6.2 Forensic evidence by direct genomic sequencing

A remarkable report was published on a rape case in which direct genomic sequencing was used to compare HIV-1 *pol* and *gag* genes sequences from the male defendant and the female victim. This case was probably the first time that evidence produced by DNA sequencing has been used in court (93, 150). The direct sequence analysis using solid-phase DNA sequencing showed that the virus populations harboured by the male and the female were highly homologous.
This study demonstrates the power of direct genomic sequencing in forensic medicine (93).

1.7.7 Large-scale DNA sequencing

The increasing demand for reliable, automated procedures for large-scale sequencing projects (e.g. Human Genome Project) has led to several methods being proposed for integrating template purification, sequencing reactions, product separation and detection (151, 152).
Uhlén *et al.* (152) have developed a solid-phase, semi-automated system for large-scale sequencing using commercially available robots and instrumentation to perform all steps in sequencing, including template preparation, sequencing reactions and the subsequent electrophoresis. The only manual operation is limited to transfer of microtiter plates between workstations. This system demonstrates that the automated solid-phase protocol using a commercially available fluorescent detection system provides a method where the running costs (reagents, enzymes, label etc.) are in a range acceptable for large-scale genome projects (151).

Rolfs *et al.* (153) have recently developed a fully-automated, nonradioactive solid-phase sequencing protocol of genomic PCR products. It uses Dynabeads M-280 Streptavidin as solid support and a computer-controlled device (PolySeq™) with heating, magnetic and mixing functions integrated in a robotic workstation (Biomek™ 1000). This solid-phase method is extremely useful for template purification and strand separation of DNA obtained from PCR. Compared with double-stranded DNA sequencing, this method yields consistently better results with increased lengths of readable sequences and reduced backgrounds.
This fully-automated sequencing method affords highly standardized sample handling, eliminates pipetting errors, and allows secure and efficient sample processing.
The system allows the complete automation of the sequencing procedure starting with PCR amplicons with an analysis capacity of 30,000–40,000 bp per week (153).

The solid-phase method using Dynabeads is also used in an international effort to determine the complete nucleotide sequence of the genome of *E.coli* K-12 (154, 155).
The strategy is based on transposon-containing clones to provide universal primers binding sites for the PCR and subsequent automated DNA sequencing.

Robots which enable PCR, magnetic separation, liquid handling and sample loading tailor-made for routine clinical applications and large-scale sequencing projects are currently under development by several instrument manufacturers.

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2. mRNA ISOLATION USING DYNABEADS® OLIGO $(dT)_{25}$

2.1 General introduction

Dynabeads Oligo $(dT)_{25}$ is designed for **rapid** isolation of highly purified, intact poly A^+ mRNA from eukaryotic **total RNA** or **directly** from crude extracts of animal tissues, cells and plants.

The direct poly A^+ mRNA isolation is performed in **15 minutes** without having to perform any preliminary purification steps (1, 2). A strong RNase inhibiting agent together with stringent hybridization and washing conditions assure the isolation of highly purified, intact poly A^+ mRNA even from crude samples rich in RNases (3, 4, 5). A schematic flow-chart for mRNA isolation from different starting materials is shown in Figure 2.1.

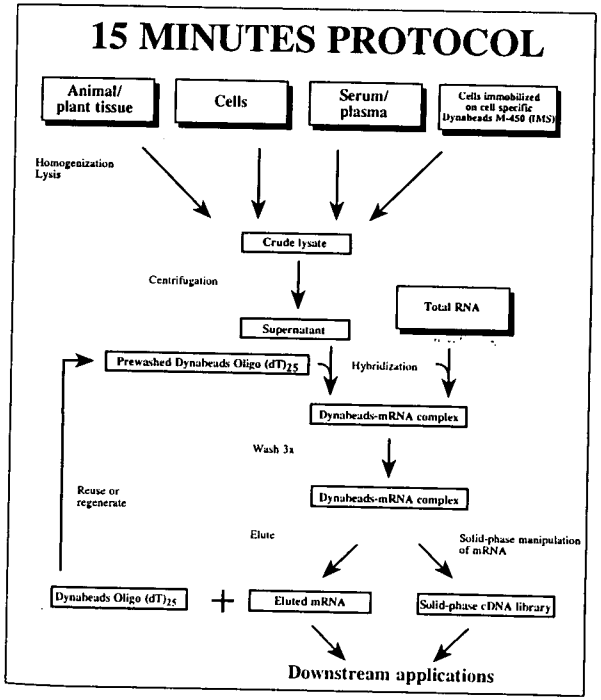

Figure 2.1 Schematic flow-chart for mRNA isolation from different starting materials using Dynabeads Oligo $(dT)_{25}$

The isolated poly A^+ mRNA may be eluted in volumes down to 5 µl and used directly in all downstream applications in molecular biology (6) including the following:

- cDNA synthesis
- Solid-phase cDNA library
- RT-PCR
- Subtractive hybridization
- Northern blotting
- S1 nuclease analysis
- Ribonuclease protection
- Primer extension
- Dot and slot hybridization
- *In vitro* translation

The isolated poly A^+ mRNA is ideal for gene cloning and gene expression analysis. Enzymatic downstream applications are not inhibited by the presence of the Dynabeads Oligo $(dT)_{25}$.

For more information on immobilized (solid-phase) cDNA library, RT-PCR and subtractive hybridization using Dynabeads, see chapter 3 and 4.

Dynabeads Oligo $(dT)_{25}$ eliminates the need for an intermediate total RNA purification step, non-flexible spin columns, laborious filtration, hazardous organic reagents and time-consuming ethanol precipitation. Further, only a minute amount of starting material is required as the Dynabeads hybridization is efficient and takes place with reaction kinetics comparable to those in free solution.

High quality mRNA purification may be performed conveniently in a single tube, and the method can be scaled up or down to suit the user's needs. See Table 2.4 in section 2.5 for small and large scale, direct mRNA isolation.

The reusable properties of the Dynabeads® Oligo $(dT)_{25}$ provides a cost effective choice for the user.

Poly A^+ mRNA has been isolated directly from the following sample types:

- Animal tissues
- Plant tissues
- Cells
- Sera and plasma
- Immobilized cells purified by immunomagnetic separation (IMS)
- Paraffin-embedded tissues

Poly A^+ mRNA has been isolated directly from highly RNase-rich materials such as liver, spleen, monocytes, and macrophages (3, 4, 5), complex tissues like human cartilage and paraffin-embedded tissue (7, 8), single *Drosophila* flies (5), and down to one cell samples (9).

Dynal's three main protocols for mRNA isolation are listed in Table 2.1A together with recommended Dynal products.

The two first, **main** protocols for **direct** mRNA isolation from solid tissues and cells use a LiDS/LiCl based lysis buffer (section 2.3.1 and 2.3.2). The RNA is stable for more then **30 minutes** at room temperature in this lysis buffer without being degraded by RNases (5). Note that the direct mRNA isolation procedure is performed in only **15 minutes.**

Using the LiDS/LiCl lysis buffer system, the Dynabeads Oligo $(dT)_{25}$ can be regenerated up to four times (section 2.4).

A lysis/denaturing buffer system based on the chaotropic reagent guanidine thiocyanate (GTC) may be used instead of the supplied LiDS/LiCl lysis buffer. However, a dilution step is then required to reduce the viscosity of lysate and to obtain proper hybridization conditions during mRNA isolation. GTC is not recommended if regeneration of the Dynabeads Oligo $(dT)_{25}$ is desired, due to loss of Dynabeads and difficulty of handling the Dynabeads after regeneration.

Dynal recommends using the LiDS/LiCl method, as it gives a higher yield and has a greater tolerance with respect to the various tissues and cells compared to using a GTC protocol (5). For further information on LiDS and GTC, please see section 2.5.

The third main protocol (section 2.3.3) in Table 2.1A provides a rapid and easy procedure for purification of mRNA from total RNA.

Table 2.1A Main Dynal protocols and products for poly A^+ mRNA isolation from different sample types.

Sample types	Dynal products	Dynal protocols
Animal and plant tissues	Dynabeads mRNA DIRECT™ Kit	Section 2.3.1
Cells	Dynabeads mRNA DIRECT Kit	Section 2.3.2
Total RNA	Dynabeads mRNA Purification Kit	Section 2.3.3

The **Dynabeads Oligo $(dT)_{25}$** are available as two ready-to-use kits; **Dynabeads mRNA DIRECT kit** and **Dynabeads mRNA Purification kit**, and two individual products; **Dynabeads Oligo $(dT)_{25}$** (2 x 1 ml) and **Dynabeads Oligo $(dT)_{25}$** (5 x 1 ml) to be used with customer-made buffers.

Three additional protocols are listed in Table 2.1B.

The first **additional** protocol (section 2.3.4) is a combined cell- and mRNA isolation protocol, for direct mRNA isolation from cell specific leukocytes immobilized and purified by immunomagnetic separation (IMS). This protocol may be used if direct mRNA isolation from subpopulations of blood cells is desired, or as a guideline for direct mRNA isolation from other specific cell types. The enclosed protocol is a powerful approach to gene regulation studies (see section 3.3.5). It enables a very rapid isolation of specific cells which may be lysed while still attached to the Dynabeads. For more information on immunomagnetic separation (IMS) please see chapter 8.

The second additional protocol (section 2.3.5) is an alternative cell protocol, and may be used for mRNA isolation from lymphocytes. This protocol uses a mild, non-ionic detergent NP-40 to immediately disrupt the cell membrane without disrupting the nuclei. The nuclei may then be removed by microcentrifugation and the mRNA is recovered directly from the nuclei (DNA) free, cytoplasmic supernatant. Removing the nuclei (DNA), will reduce the viscosity of lysate and improve the hybridization efficiency during mRNA isolation.

For more compact cell types such as HeLa cells, and RNase rich cells (monocytes and macrophages) stronger lysis and denaturing conditions are required (3, 4, 5). The main **cell** protocol (section 2.3.2) should then be used. A DNA-shear step is recommended.

The third additional protocol in Table 2.1B (section 2.3.6) is a complete protocol for highly sensitive HIV-RNA detection in sera and plasma. This is a direct protocol for rapid and easy isolation and detection of polyadenylated HIV-RNA without any preliminary purification steps.

Table 2.1B Additional Dynal protocols with recommended Dynal products for poly A^+ mRNA isolation from different sample types.

Sample types	Dynal products	Dynal protocols
Immobilized leukocytes purified by IMS	Cell specific Dynabeads M-450 and Dynabeads mRNA DIRECT Kit or Cell specific Dynabeads M-450, Dynabeads mRNA Purification kit and a lysis buffer with a mild, non-ionic detergent	Section 2.3.4
Lymphocytes	Dynabeads mRNA Purification kit and a lysis buffer with a mild, non-ionic detergent	Section 2.3.5
Sera and plasma (viral RNA)	Dynabeads mRNA DIRECT kit	Section 2.3.6

References for **direct** mRNA isolation from various crude starting materials are listed in the following tables: Table 2.2A (animal tissues), Table 2.2B (plant tissues), and Table 2.2C (cells). References for mRNA isolation from **total RNA** are listed in Table 2.3.

Materials required for mRNA isolation using Dynabeads Oligo $(dT)_{25}$ are listed in section 2.2. Buffers and solutions are listed in section 2.6.

Note, that all common buffers for mRNA purification/extraction can be used with Dynabeads Oligo $(dT)_{25}$.

Table 2.2A mRNA DIRECT from animal tissues

Tissue	Species	References
Adiposity	swine	64
[illegible]renals	rat	5
[illegible]in	mouse, rat, trout	5, 24, 25, 26, 57
Cartilage	human	7
Eggs	trout	5
Heart	rat	26
Kidney	rat	26
Liver	rat, trout, *Xenopus*	5, 26
Lung	rat	26
Muscle	trout	5
Ovaries	trout, *Xenopus*	5
Paraffin-embedded lung	human	8
Pronephros	trout	5
Spleen	rat	5, 26
Whole insect	*Drosophila*	5

Table 2.2B mRNA DIRECT from plant tissues

Tissue	Species	References
Whole plants	*Arabidopsis*	34, 62
[illegible]aves	Barley, Potato	1, 5, 56
Roots	Spruce, Barley	1, 30
Seed aleurone	Barley	1, 5, 22, 37
Seed endosperm	Barley	22, 37
Seed embryoes	Barley	1, 5, 22, 37
Stolon tips	Potato	56
Tubers	Potato	56

Table 2.2C mRNA DIRECT from different types of cells

Cell type/cell line	Origin	References
AMA	Human placental cells	3
Chondrocytes	Human	61
Daudi	Human B-cell line	36
D551	Human fibroblasts	3
Endothelial cells	Human umbilical cord	3
HepG2	Human hepatocytes	3
HeLa	Human cervical cancer cells	3
HL-60	Human	5
Hybridoma cells	Mouse	2
Keratinocytes	Human	32
Langerhans cells	Human	32
Lymphocytes	Human	35
MCF7	Human mammary carcinoma	3
Monocytes	Human	4
Peritoneal exoduate cells	Human	32
Reh	Human B-cell line	9, 36
RTG-2	Trout fibroblasts	5
SKB-1	Human fibroblasts	31
ST-1	Human fibroblasts	31
T-cells / T-cell clones	Human	27, 28, 29, 33, 36
Saccharomyces cerevisiae	Yeast	23

Table 2.3 mRNA purification from total RNA

Tissue/cells	Species	References
Adrenals	Human	3
Brain	Mouse	38
B-cells	Human	39
Blood	Human	49
Cultured cells	Human	3, 46, 47, 48, 55
[illegible]bryos	Mouse	58
[illegible] cup	Insects	54
Fibroblasts	Human	59
Heart	Rabbit	51
Intestine	Human, Rabbit	3, 51
Kidney	Rabbit	51
Liver	Human, Rabbit	3, 50, 51
Lung	Human	3, 52
Macrophages	Human	53
Mononuclear cells, tonsilla	Human	39
Muscle	Human	60
Pancreas	Human	3
Retina	Human	50
Sperm	*Abalone*	45
Spleen	Human, Rabbit	3, 51
Testis	Human	3
T-cells	Human	41, 42
Plants	*Arabidopsis*	44
[illegible]floral tissue	*Rumex acetosa* (Sorrel)	63
[illegible]aves	Tobacco	40
Roots, stems, nodes, leaves, flowers and cultured cells	*Alfalfa*	43
Root tips	Tomato	10

2.2 Materials required

- Dynabeads Oligo $(dT)_{25}$ (used with customer-made buffers)
  Note: Buffers and solutions are described in chapter 2.6
- Dynabeads mRNA DIRECT kit contains:
  Dynabeads Oligo $(dT)_{25}$
  Lysis/binding buffer
  Washing buffer with LiDS (SDS)
  Washing buffer
  Elution solution
  Reconditioning solution
  Storage buffer Oligo $(dT)_{25}$
- Dynabeads mRNA Purification kit contains:
  Dynabeads Oligo $(dT)_{25}$
  2 x Binding buffer
  Washing buffer
  Elution solution
  Dynal MPC-E-1
- Magnetic Particle Concentrator - Dynal MPC (see Appendix B).
- Liquid nitrogen
- Manual tissue grinder or homogenizer (see below)
- Syringe (see section 2.5; viscosity reduction by DNA-shearing)
- 65°C water bath or heating block
- Sterile, RNase-free Microtubes of the Eppendorf type.
- Sterile, RNase-free pipettes and pipette tips

Suitable homogenizers - A complete lysis of the cells is critical for obtaining a good yield of poly A+ mRNA directly from crude lysate. The homogenizer is an important tool in this respect. The choice of homogenizer is dependent on the particular application and tissue. The following homogenizers have been used successfully:
1) Pellet Pestle® Mixer for microtubes, Kontes Biotechnology, Vineland, NJ, USA (1)
2) Dual® glass homogenizer, Kontes Biotechnology, Vineland, NJ, USA (1)
3) PolytronR PT1200, Kinematica, Littau, Switzerland.
4) Fisher Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA. (10)

The Pellet Pestle Mixer is designed to grind very soft tissue. Glass homogenizers (e.g. Dual glass homogenizer) are available in two different forms. The ground glass to ground glass form is best suited for tough, connective tissues such as heart and lung. The teflon pestle to smooth glass form is used for homogenizing soft tissues such as brain and liver. The Polytron acts more by a rapid chopping action compared to traditional tissue grinders which act by shearing and compression. Some use a Polytron style homogenizer for the initial grind and continues with a glass homogenizer. The Polytron may be used alone to produce a raw extract provided the grinding time is kept short due to foaming of the detergent in the lysis buffer. For small volumes the Polytron 1200 is recommended.
RNase treatment of glass homogenizers
Make sure your homogenizer is free from RNases before use. Glass homogenizers may be RNase-treated by placing them in 0.1% diethyl pyrocarbonate (DEPC) for 1 h and then autoclaved.

2.3 Protocols

2.3.1 Direct poly A+ mRNA isolation from crude animal tissues and plants using the Dynabeads mRNA DIRECT kit (1, 5)

This protocol is recommended for direct, high purity, intact poly A+ mRNA isolation from various crude tissue samples (Figure 2.2). The Dynabeads mRNA DIRECT kit provides a LiDS/LiCl based lysis buffer in which RNA is stable for 30 minutes at room temperature without being degraded by RNases (5). Dynal recommends using the LiDS/LiCl method, as it gives a higher yield and has a greater tolerance with respect to the various tissues and cells compared to using a GTC protocol (5). The Dynabeads mRNA DIRECT procedure is very flexible and may be scaled up or down to suit your own experimental design (section 2.5, Table 2.4). Using

the LiDS/LiCl lysis buffer system, Dynabeads Oligo $(dT)_{25}$ can be regenerated up to four times (section 2.4).

Conditioning of the Dynabeads Oligo $(dT)_{25}$

1. Resuspend the Dynabeads Oligo $(dT)_{25}$ thoroughly before use.
2. Transfer Dynabeads Oligo $(dT)_{25}$ (0.25 ml) from the stock tube suspension to an RNase-free Eppendorf tube placed in a Dynal MPC (Dynal MPC-E/Dynal MPC-M)
3. After 30 s (or when the suspension is clear) remove the supernatant.
   Note: Do not leave the Dynabeads unsuspended for a long period of time, as drying of the Dynabeads may lower their capacity.
4. Remove the vial from the Dynal MPC and prewash the Dynabeads Oligo $(dT)_{25}$ by resuspending in **Lysis/binding buffer** (0.2 ml). Again place the vial in the Dynal MPC.

Preparation of lysate from crude animal tissues and plants

5. Grind frozen animal tissue (20-50 mg) or plant tissue (100 mg) in liquid nitrogen.
   Note: Work quickly. Aliquot (weigh) the animal or plant tissue while frozen, to avoid mRNA degradation. Please, use the specified amount of tissue, since an excess of tissue will reduce the mRNA yield and purity (5).
6. Transfer the frozen powder to a homogenizer (section 2.2) containing **Lysis/binding buffer** (1.0 ml) and homogenize until complete lysis is obtained (approx. 1-2 min).
   Note: A rapid lysis in the **Lysis/binding buffer** is critical for obtaining undegraded mRNA.
7. Spin the lysate for 30-60 s in an Eppendorf centrifuge. Transfer the supernatant to a new vial to remove debris.
8. Reduce the viscosity by a DNA-shear step. The lysate is pressed three times through a 21 gauge needle by a 1-2 ml syringe (use force). The reduced viscosity should be visible.
   Note: The lysate can be frozen in liquid nitrogen and stored for later use.

Direct mRNA isolation from crude lysate

9. Remove the **Lysis/binding buffer** from the Dynabeads Oligo $(dT)_{25}$ vial already placed in the Dynal MPC (see step 4). Transfer the vial from the Dynal MPC to another rack, and add the lysate.
10. Mix the Dynabeads Oligo $(dT)_{25}$ with the lysate and anneal by rotating on a roller for 3-5 min at room temperature.
    Note: Increase the annealing time if the solution is viscous.
11. Place the vial in the Dynal MPC for 2 min and remove the supernatant. Transfer the vial to another rack.
    Note: If the solution is noticeably viscous, increase the time to approx. 5 min.
12. Wash twice with **Washing buffer with LiDS** (0.5-1 ml) at room temperature using the Dynal MPC and once with **Washing buffer** (0.5 ml).
    Note: The Dynabeads must be mixed thoroughly in the washing buffer. Make sure to remove the supernatant completely between each washing step.
    For enzymatic downstream applications
    If the isolated mRNA is to be used in enzymatic downstream applications, wash two extra times with **Washing buffer** (0.5 ml) or with the enzymatic buffer to be used (chapter 3).
13. If elution of mRNA from the Dynabeads is necessary, add **Elution solution** (10-20 µl) and keep at 65°C for 2 min. Place the tube in the Dynal MPC and transfer the supernatant containing the mRNA to a new RNase-free tube.
    Note: The final yield may vary somewhat between tissues depending on mRNA abundance.
    For Northern blotting the mRNA can be eluted directly in the loading buffer or in TE/Elution solution. Dynabeads may be regenerated/washed and reused four times (section 2.4).
    It is also possible to perform solid-phase cDNA synthesis on the Dynabeads Oligo $(dT)_{25}$ (chapter 3).

Figure 2.2 **Direct mRNA isolation from a variety of crude samples.**
Northern blot analysis of mRNA from 50 mg solid tissue using β-actin as probe. Lane 1-5: Fish liver, kidneys, brain, small and large roe, lane 6: rat liver, lane 7: fish cells (stimulated), lane 8: fish cells (non-stimulated), lane 9: rat adrenal gland, lane 10: Xenopus ovaries. Courtesy of K. Jakobsen and M. Haugen, Univ. of Oslo, Norway (5).

2.3.2 Direct poly A^+ mRNA isolation from cells using the Dynabeads mRNA DIRECT kit (3)

This protocol is recommended for all cell types. In particular for compact cells (e.g HeLa) and RNase rich cells (monocytes, macrophages) which require strong lysis and denaturing conditions. The Dynabeads mRNA DIRECT kit uses a LiDS/LiCl lysis buffer in which RNA is stable for 30 minutes at room temperature without being degraded by RNases (5). Dynal recommends using the LiDS/LiCl method, as it gives a higher yield and has a greater tolerance with respect to the various tissues and cells compared to using a GTC protocol (5). The Dynabeads mRNA DIRECT procedure is very flexible and may be scaled up or down to suit your own experimental design (section 2.5, Table 2.4). Using the LiDS/LiCl lysis buffer system, the Dynabeads Oligo $(dT)_{25}$ may be regenerated up to four times (section 2.4).

For lymphocytes and certain tissue culture cells an alternative protocol is possible (section 2.3.5). The nuclei may then be removed without releasing the DNA.

Conditioning of the Dynabeads Oligo $(dT)_{25}$

1. Resuspend the Dynabeads Oligo $(dT)_{25}$ thoroughly before use.
2. Transfer Dynabeads Oligo $(dT)_{25}$ (0.25 ml) from the stock tube suspension to an RNase-free Eppendorf tube placed in a Dynal MPC (Dynal MPC-E/ Dynal MPC-M).
3. After 30 s (or when the suspension is clear) remove the supernatant.
   Note: Do not leave the Dynabeads unsuspended for a long period of time, as drying of the Dynabeads may lower their capacity.
4. Remove the vial from the Dynal MPC and prewash the Dynabeads Oligo $(dT)_{25}$ by resuspending in **Lysis/binding buffer** (0.2 ml). Again place the vial in the Dynal MPC.

Preparation of crude cell lysate

5. Wash the cell suspension in phosphate-buffered saline (1 x PBS) prior to preparing a cell pellet by centrifugation. The cell pellet can be used immediately, or frozen in liquid nitrogen.
6. Add **Lysis/binding buffer** (1.0 ml) to either a frozen cell pellet or to a fresh cell pellet (1-4 x 10^6). Perform a repeated passage of the solution through a pipette tip to obtain complete lysis.
   Note: The release of DNA during lysis results in a viscous solution which confirms a complete lysis.
7. Reduce the viscosity by a DNA-shear step. The lysate is pressed three times through a 21 gauge needle by a 1-2 ml syringe (use force). The reduced viscosity should be visible.
   Note: Repeated shearing causes foaming of the lysate due to detergent in the buffer. However, this should not effect the mRNA yield. The foam can be reduced by a 30 s centrifugation.
   The lysate may be frozen (- 80°C) and stored for later use.

Direct mRNA isolation from crude cell lysate

8. Remove the **Lysis/binding buffer** from the Dynabeads Oligo (dT)$_{25}$ vial already placed in the Dynal MPC (see step 4). Transfer the vial from the Dynal MPC to another rack, and add the lysate.

9. Mix the Dynabeads Oligo (dT)$_{25}$ with the lysate and anneal by rotating on a roller for 3-5 min at room temperature.
   Note: Increase the annealing time if the solution is viscous.

10. Place the vial in the Dynal MPC for 2 min and remove the supernatant.
    Note: If the solution is noticeably viscous, increase the time to approx. 5 min.

11. Wash twice with **Washing buffer with LiDS** (0.5-1 ml) at room temperature using the Dynal MPC and once with **Washing buffer** (0.5 ml).
    Note: The Dynabeads must be mixed thoroughly in the washing buffer. Make sure to remove the supernatant completely between each washing step.
    For enzymatic downstream applications
    If the isolated mRNA is to be used in enzymatic downstream applications, wash two extra times with **Washing buffer** (0.5 ml) or with the enzymatic buffer to be used (chapter 3).

12. If elution of mRNA from the Dynabeads is necessary, add **Elution solution** (10-20 µl) and keep at 65°C for 2 min. Place the tube in the Dynal MPC and transfer the supernatant containing the mRNA to a new RNase-free tube.
    Note: The final yield may vary somewhat between cells depending on mRNA abundance.
    For Northern blotting the mRNA can be eluted directly in the loading buffer or in TE/Elution solution. Dynabeads may be regenerated/washed and reused four times (section 2.5).
    It is also possible to perform solid-phase cDNA synthesis on the Dynabeads Oligo (dT)$_{25}$ (chapter 3).

2.3.3 Poly A$^+$ mRNA isolation from total RNA preparations using the Dynabeads mRNA Purification kit

This protocol is designed for rapid and easy purification of mRNA from total RNA preparations (Figure 2.3). The procedure is very flexible and may be scaled up or down to suit your own experimental design.

Conditioning of Dynabeads Oligo (dT)$_{25}$

1. Resuspend the Dynabeads Oligo (dT)$_{25}$ thoroughly before use.
2. Transfer Dynabeads Oligo (dT)$_{25}$ (0.2 ml) from the stock tube suspension to an RNase-free Eppendorf tube placed in a Dynal MPC (Dynal MPC-E/ Dynal MPC-M).
3. After 30 s (or when the suspension is clear) remove the supernatant.
   Note: Do not leave the Dynabeads unsuspended for a long period of time, as drying of the Dynabeads may lower their capacity.
4. Remove the vial from the Dynal MPC and prewash the Dynabeads by resuspending in **2 x Binding buffer** (0.1 ml). Again place the vial in the Dynal MPC.

Preparation of total RNA

5. Adjust the volume of 75 µg RNA to 100 µl with distilled DEPC treated water or with **Elution solution**. This step can be omitted if only a small adjustment is needed.
   Note: If the total RNA (75 µg) has already been diluted in more than 100 µl please see the note under step 7. Optimal hybridization conditions are obtained in 1 x Binding buffer.

6. Heat to 65°C for 2 min.

mRNA isolation from total RNA

7. Remove the **2 x Binding buffer** from the Dynabeads Oligo (dT)$_{25}$ (see step 4). Transfer the vial to another rack and resuspend the Dynabeads in **2 x Binding buffer** (100 µl).
   Note: If the total RNA (75 µg) has already been diluted in more than 100 µl then simply add the same total volume of **2 x Binding buffer** to the Dynabeads Oligo (dT)$_{25}$ to obtain a 1 x Binding buffer concentration. This will provide the most optimal hybridization conditions.

8. Add the total RNA to the Dynabeads suspension. Mix the Dynabeads Oligo (dT)$_{25}$ thoroughly with the RNA and anneal by rotating on a roller for 3-5 min at room temperature.

9. Place the vial in the Dynal MPC for 30 s and remove the supernatant. Transfer the vial to another rack.

10. Wash twice with **Washing buffer** (0.2 ml) using the Dynal MPC
    Note: The Dynabeads must be mixed thoroughly in the washing buffer. Make sure to remove the supernatant completely between each washing step.
    For enzymatic downstream applications
    If the isolated mRNA is to be used in enzymatic downstream applications, wash once with the enzymatic buffer (0.2 ml) to be used (chapter 3).

11. If elution of mRNA from the Dynabeads is necessary, add **Elution solution** (10-20 µl) and keep at 65°C for 2 min. Place the tube in the Dynal MPC and transfer the supernatant containing the mRNA to a new RNase-free tube.
    Note: For Northern blotting the mRNA can be eluted directly in the loading buffer or in TE/Elution solution. Dynabeads may be regenerated/washed and reused four times (section 2.4).
    It is also possible to perform solid-phase cDNA synthesis on the Dynabeads Oligo (dT)$_{25}$ (chapter 3).

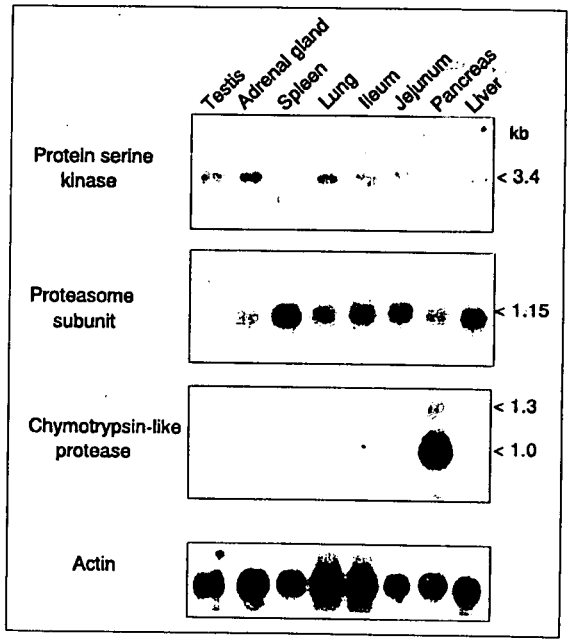

Figure 2.3
Purification of mRNA from total RNA. Northern blot of mRNA from 8 human tissues. mRNA isolated with Dynabeads Oligo (dT)$_{25}$ from total 20 µg total RNA in each lane. The blot was hybridized successively with probes from a protein serine kinase gene, a proteasome subunit gene, and a chymotrypsin-like gene and finally with a beta-actin probe as a control. The size of the transcripts are shown on the left as calculated from a standard of denaturated DNA fragments. Courtesy of F. Larsen *et al.* (3).

2.3.4 Immunomagnetic separation (IMS) of specific leukocytes combined with direct mRNA isolation using the Dynabeads mRNA DIRECT kit (11)

This protocol enables a very rapid isolation of specific cells types, which may be lysed while still attached to the Dynabeads (IMS is described in chapter 8). The described method may be used if direct mRNA isolation from subpopulations of blood cells is desired or as a guideline for direct mRNA isolation from other specific types of cells. The protocol is a powerful approach to gene regulation studies (results are shown in section 3.3.5).

Note that many of the nucleated blood cells have a low content of mRNA. This protocol is suitable for RT-PCR amplification of transcripts. Further, for lymphocytes and certain tissue culture cells an alternative poly A+ mRNA isolation protocol is possible (section 2.3.5). The nuclei may then be removed without releasing the DNA.

Immunomagnetic separation and immobilization of leukocytes from citrate blood using cell specific Dynabeads M-450

Note: Dynabeads M-450 CD 14, CD 15, CD 19, CD 2 and CD 15+45 products have been used successfully for this immunomagnetic separation of leukocytes, which is combined with direct mRNA isolation and subsequent RT-PCR (section 3.3.5) (11).

1. Cool 0.5 ml citrate blood (ACD) for minimum 3 min (approx. 4–10 x 10^6 leukocytes/ml).
2. Resuspend the cell specific Dynabeads M-450 thoroughly before use. Prewash 50 µl (2 x 10^7) Dynabeads M-450 in 1 x PBS/citrate (500 µl). Collect the Dynabeads using a Dynal MPC (Dynal MPC-E) and remove the 1 x PBS/citrate. Add the blood sample (0.5 ml) to the Dynabeads and mix gently.
3. Incubate the tube by rotating on a roller for 20 min at 4°C to immobilize the leukocytes on the Dynabeads.
   Note: For monocytes, 10 min immobilization time at 4°C is sufficient.
4. Place the tube in a magnetic stand for 3 min and remove the supernatant.
5. Wash the isolated leukocyte/Dynabeads M-450 complex four times in cold 1 x PBS/citrate (500 µl). Transfer the complex to a new Eppendorf tube at the last washing step. Collect the Dynabeads with the magnet between each washing step. Remove the washing buffer after the last washing and proceed immediately with the mRNA isolation.

Direct poly A+ mRNA isolation from immobilized leukocytes

6. Add **Lysis/binding buffer** (700 µl) to the leukocyte/Dynabeads M-450 complex. Mix well to obtain complete lysis.
7. Resuspend the Dynabeads Oligo $(dT)_{25}$ thoroughly before use.
8. Transfer 300 µg Dynabeads Oligo $(dT)_{25}$ (60 µl) from the stock tube suspension to an RNase-free Eppendorf tube placed in a Dynal MPC (Dynal MPC-E).
9. After 30 s (or when the suspension is clear) remove the supernatant.
   Note: Do not leave the Dynabeads unsuspended for a long period of time, as drying of the Dynabeads may lower their capacity.
10. Remove the vial from the Dynal MPC and prewash the Dynabeads Oligo $(dT)_{25}$ by resuspending in **Lysis/binding buffer** (200 µl). Again place the vial in the Dynal MPC.
11. Place the tube with the lysate in the magnetic stand (Dynal MPC-E) for 3 min to collect the Dynabeads M-450.
12. Remove the **Lysis/binding buffer** from the Dynabeads Oligo $(dT)_{25}$ vial already placed in the Dynal MPC. Transfer the vial from the Dynal magnet to another rack, and add the cell lysate from step 11 (without the Dynabeads M-450).

13. Mix the Dynabeads Oligo $(dT)_{25}$ with the lysate and place the vial on a roller for 3-5 min at room temperature to let the polyadenylated mRNA anneal to the Dynabeads Oligo $(dT)_{25}$.
14. Place the vial in the Dynal MPC for 2 min and remove the supernatant.
15. Wash twice with **Washing buffer with LiDS** (0.5 ml) at room temperature using the Dynal MPC and once with **Washing buffer** (0.5 ml). Resuspend the Dynabeads in **Washing buffer** and place the vial on ice.
    Note: The Dynabeads must be mixed thoroughly in the washing buffer. Make sure to remove the supernatant completely between each washing step.
    For enzymatic downstream applications
    If the isolated mRNA is to be used in enzymatic downstream applications, wash two extra times with **Washing buffer** (0.5 ml) or with the enzymatic buffer to be used (RT-PCR is described in chapter 3).
16. If elution of mRNA from the Dynabeads is necessary, add **Elution solution** (10–20 µl) and keep at 65°C for 2 min. Place the tube in the Dynal MPC and transfer the supernatant containing the mRNA to a new RNase-free tube.
    Note: The final yield may vary somewhat between cells depending on mRNA abundance.
    The Dynabeads may be regenerated/washed and reused four times (section 2.4).
    It is also possible to perform solid-phase cDNA synthesis on the Dynabeads Oligo $(dT)_{25}$ (chapter 3).

2.3.5 Direct isolation of cytoplasmic poly A+ mRNA from nuclei free cell lysate using Dynabeads mRNA Purification kit and a mild non-ionic detergent (9)

This protocol is recommended for lymphocytes and certain tissue culture cells. Using a mild, non-ionic detergent NP-40 the cell membrane will disrupt immediately without disrupting the nuclei. The nuclei are removed by microcentrifugation and mRNA is recovered directly from the nuclei free, cytoplasmic supernatant. Removing the nuclei (DNA) will reduce the viscosity of lysate and improve the hybridization efficiency during mRNA isolation.

For more compact cells such as HeLa cells, and RNase rich cells (monocytes and macrophages) stronger lysis and denaturing conditions are required (3, 4, 5). The main Dynal protocol for cells is then recommended (section 2.3.2).

Conditioning of Dynabeads Oligo $(dT)_{25}$

1. Resuspend the Dynabeads Oligo $(dT)_{25}$ thoroughly before use.
2. Transfer Dynabeads Oligo (dT)*25* (350–450 µg Dynabeads/10^6 cells depending on tissue) from the stock tube suspension to an RNase-free Eppendorf tube placed in a Dynal MPC.
3. After 30 s (or when the suspension is clear) remove the supernatant.
   Note: Do not leave the Dynabeads unsuspended for a long period of time, as drying of the Dynabeads may lower their capacity.
4. Remove the vial from the Dynal MPC and prewash the Dynabeads Oligo $(dT)_{25}$ by resuspending in **2 x Binding buffer** (0.2 ml). Again place the vial in the Dynal MPC.

Preparation of nuclei-free cell lysate

5. Wash the cells (1–3 x 10^6) in ice cold phosphate-buffered saline (1 x PBS) prior to preparing a cell pellet by centrifugation.
6. Resuspend the pellet in 100 µl Lysis buffer with NP-40 (1% NP-40,10 mM Tris-HCl (pH 7.5), 0.14 M NaCl, 5 mM KCl). Place on ice for 1 min.
7. Centrifuge for 30 s.

mRNA isolation from nuclei-free cell lysate

8. Remove the **2 x Binding buffer** from the Dynabeads Oligo $(dT)_{25}$ already placed in the Dynal MPC (see step 4). Transfer the vial to another rack and add **2 x Binding buffer** (100 µl).
9. Add the cell lysate to the Dynabeads Oligo $(dT)_{25}$ already resuspended in 100 µl **2 x Binding buffer.**
10. Mix the Dynabeads Oligo $(dT)_{25}$ thoroughly with the lysate and anneal by rotating on a roller for 3-5 min at room temperature.
11. Place the vial in the Dynal MPC for 2 min and remove the supernatant. Transfer the vial to another rack.
12. Wash twice with **Washing buffer** (0.2 ml) using the Dynal MPC

Note: The Dynabeads must be mixed thoroughly in the washing buffer. Make sure to remove the supernatant completely between each washing step.
For enzymatic downstream applications
If the isolated mRNA is to be used in enzymatic downstream applications, wash once with the enzymatic buffer (0.2 ml) to be used (chapter 3).

13. If elution of the mRNA from the Dynabeads is necessary, add **Elution solution** (10–20 µl) and keep at 65°C for 2 min. Place the tube in the Dynal MPC and transfer the supernatant containing the mRNA to a new RNase-free tube.

Note: For Northern blotting the mRNA can be eluted directly in the loading buffer or in TE/Elution solution. The Dynabeads may be regenerated/washed and reused four times (section 2.4).
It is also possible to perform solid-phase cDNA synthesis on the Dynabeads Oligo $(dT)_{25}$ (chapter 3).

2.3.6 Protocol for highly sensitive detection of polyadenylated HIV-RNA in sera and plasma using the Dynabeads mRNA DIRECT kit (12-16)

Isolation and detection of polyadenylated and non-polyadenylated viral RNA

Methods have been described for the use of Dynabeads Oligo $(dT)_{25}$ to isolate polyadenylated viral RNA, e.g. HIV-1 RNA from cerobrospinal fluid (14) or serum (15). Disruption of viruses and cells is obtained with the detergent LiDS or SDS. These are RNase inhibitors. However, RNase inhibitors such as RNAguard® or Vanadyl Ribonucleosyl Complexes may also be used.

The method recommended describes the use of the Dynabeads mRNA DIRECT kit to isolate viral polyadenylated RNA by using the detergent LiDS. RNA is stable for at least 30 minutes in the lysis buffer at room temperature (5) and with this rapid purification protocol (15 minutes) high quality RNA is ensured.

The method is a highly sensitive assay for detection of HIV-1 RNA in serum and plasma (Figure 2.4). Recently, a similar protocol, has been successfully used for detection of HIV-2 RNA and HTLV-I RNA, suggesting that this extraction protocol may also be suitable for isolation of other polyadenylated viral RNAs (13).

When the viral RNA is not polyadenylated, the poly (A) tail is not readily available or the viral genome is DNA (ss), Dynabeads M-280 Streptavidin and specific biotinylated probes may be used instead of Dynabeads Oligo $(dT)_{25}$ using the same extraction protocol. With such an extraction protocol, RNA of poliovirus and influenzavirus (which has a segmented not polyadenylated RNA genome) has been successfully isolated (13). Dynabeads M-280 Streptavidin with a specific biotinylated probe to isolate viral RNA have been reported, e.g. enterovirus (17) (chapter 5).

For detection of the isolated viral RNA, a reverse transcription to cDNA is performed followed by a specific PCR amplification of a well known region of the viral RNA to be detected (RT-PCR). Conventional methods for direct detection of the virus include cocultivation with a susceptible cell line and/or detection of viral antigens. Although culture techniques have been greatly improved, the procedure is laborious, time-consuming, and costly and requires the handling of large amounts of infectious agent. The use of a nested PCR approach provides a highly sensitive and specific alternative for direct detection of HIV-1.

The method performs the RT-PCR in solution with eluted mRNA. There are similar methods that uses a solid-phase RT-PCR approach (chapter 3) (13). For the specific detection, all methods use a nested PCR amplification.

HIV-1 and HIV-2 can be detected as free viruses in plasma and serum. HTLV-I seems to be strictly cell associated and HTLV-I detection by PCR has to be performed with mononuclear cells from blood, or with cultured virus from such cells. Because it takes several weeks before a culture becomes positive with HTLV-1 antigens in ELISA, the present protocol followed by nested primer RT-PCR (npRT-PCR) may improve the early detection of virus in the culture supernatant.

In conclusion, the Dynabeads Oligo $(dT)_{25}$ method using LiDS/LiCl is found to be highly sensitive for detection of polyadenylated viral RNA in serum and plasma.

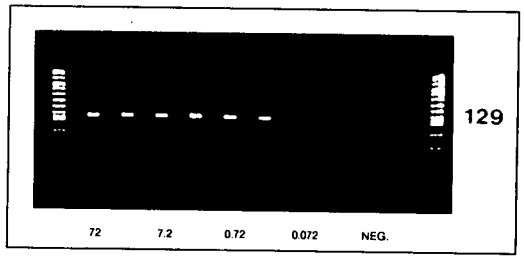

Figure 2.4
Analysis of sensitivity level for HIV-1 detection using Dynabeads mRNA DIRECT kit and solid-phase npRT-PCR.
Duplicate plasma samples of each dilution step from 72 to 0.072 $TCID_{50}$ (50% Tissue Culture Infectious Dose) HIV-1. 0.72 $TCID_{50}$ HIV-1 is equivalent to approximately 1 to 10 genomic RNA copies. Direct HIV-1 RNA isolation was performed with 50 µl Dynabeads Oligo $(dT)_{25}$ and 250 µl sample. Primers from the *pol* gene of HIV-1 were used for the solid-phase npRT-PCR assay. Weight marker was marker VIII from Boehringer Mannheim. Courtesy of A. Meijer (13).

Protocol for highly sensitive detection of polyadenylated HIV-RNA in sera and plasma using the Dynabeads mRNA DIRECT kit (12-16)

This protocol provides a direct procedure for rapid and easy detection of polyadenylated HIV-RNA without any preliminary purification steps.

1. Mix sera (100 µl) or plasma (100 µl) with **Lysis/binding buffer** (300 µl) in an RNase free Eppendorf tube.

Note: To avoid RNAse degradation of the starting material prior to the addition of the **Lysis/binding buffer,** please work quickly and keep everything cold at 0 - 4°C. If some of the buffers show precipitation, heat and mix the buffer until the precipitate is dissolved and cool on ice before use.

2. Remove Dynabeads Oligo $(dT)_{25}$ (25 µl) from the stock tube suspension stored in phosphate-buffered saline (PBS) or from the suspension of reconditioned Dynabeads stored in **Storage buffer Oligo $(dT)_{25}$** to an RNase-free Eppendorf tube placed in a Dynal MPC-E or Dynal MPC-M. After 30 s (or when the suspension is clear) remove the supernatant, and wash once with **Lysis/binding buffer** (0.2 ml). Again remove the supernatant using the Dynal MPC magnet.

Note: Do not leave the Dynabeads unsuspended for a long period of time as drying of the Dynabeads may lower their capacity.

3. Add the conditioned Dynabeads to the supernatant of the lysate, mix, and allow the polyadenylated HIV-RNA to bind for 10 min at room temperature.
4. Place the tube in the Dynal MPC for 2 min and remove the supernatant.

Note: Make sure the supernatant is removed completely before proceeding any further.

5. Wash three times with **Washing buffer with LiDS** (0.5 ml) at room temperature using the Dynal MPC and once with **Washing buffer** (0.5 ml).

Note: The Dynabeads must be mixed thoroughly in the washing buffer. Make sure to remove the supernatant completely between each washing step.
For enzymatic downstream applications
If the isolated mRNA is to be used in enzymatic downstream applications, wash two extra times with **Washing buffer** (0.5 ml) or with the enzymatic buffer to be used.

6. Remove the **Washing buffer** using the Dynal MPC. Change to a new Eppendorf tube before performing a last washing step with **Washing buffer** (0.5 ml)
7. Add **Elution solution** (25 µl) and keep at 65°C for 2 min. Place the tube in the Dynal MPC and transfer the supernatant containing the mRNA to a new RNase-free tube. The eluate may be immediately used for reverse transcription.

Note: The mRNA/Dynabead complex may be used directly for the cDNA synthesis without performing the elution step (chapter 3) (13). However, the complex should be reconditioned in the appropriate RT-buffer prior to the transfer to the reverse transcription mixture containing the HIV-RT specific primer.

Reverse transcription assay:
JA20 and JA96 are complementary to regions in the HIV-1 *pol* and *gag* regions respectively (see below), and may be used for the reverse transcription with M-MLV.

Reverse transcription mixture (final volume 30 µl):
Isolated mRNA
50 mM Tris-HCl pH 8.3
8 mM $MgCl_2$
30 mM KCl
10 mM DTT
1.7 mM dNTPs (dATP, dCTP, dGTP, dTTP)
1.0 µM primer
3 U MoMLV reverse transcriptase
16 U RNAguard
Note: Other reverse transcriptases can be used such as the AMV-RT (13). Further, thermostable reverse transcriptases may give a more robust system performing the RT at an elevated temperature.

Nested polymerase chain reaction (final volume 50 µl):
Nested-PCR reaction mixture
10 mM Tris-HCl (pH 8.3)
3.0 mM $MgCl_2$
40 mM KCl
50 mM dNTPs (dATP, dCTP, dGTP, dTTP)
0.1 µM primer
1.0 U AmpliTaq polymerase
Note: 5 µl of outer PCR amplification product should be used for the inner PCR.

Temperature cycles for the *gag* region:
(outer amplification 24 cycles, inner amplification 30 cycles)

95°C	30 s
60°C/50°C	30 s
75°C	30 s

The *gag* region 60°C, *pol* region 50°C.

Primers JA93, JA94, JA95, JA96 located in the HIV-1 ***gag*** region may be used for the cDNA synthesis and the subsequent nested PCR assay:
Outer primer pair for the HIV-1 *gag* region as follows: (Fragment size: 535 bp)
JA93, 5'-TTATCAGAAGGAGCCACCCCACAAG-3' (1318-1342)
JA96, 5'-CTCCCTGACATGCTGTCATCATTTC-3' (1828-1804)
Inner primer pair for the HIV-1 *gag* region: (Fragment size 359 bp)
JA94, 5'-ATCAATGAGGAAGCTGCAGAATGGG-3' (1408-1432)
JA95, 5'-biotin-GGACCAACAAGGTTTCTGTCATCC-3' (1742-1718)

Primers JA17, JA20, JA79 and JA91 located in the ***pol*** gene of the HIV-1 genome may also be used for the RT-PCR assay:
Outer primer pair for the HIV-1 *pol* region: (Fragment size: 266 bp)
JA 17, 5'-TACAGGAGCAGATGATACAG-3' (2431-2450)
JA 20, 5'-CCTGGCTTTAATTTTACTGG-3' (2697-2678)
Inner primer pair for the HIV-1 *pol* region: (Fragment size: 255 bp)
JA79, 5'-AGGAGCAGATGATACAGTATTAG-3' (2598-2574)
JA91, 5'biotin-AATTTTACTGGTACAGTTTCAATAGG-3' (2598-2574)

Nucleotide positions are given relative to the MN strain of HIV-1 (18).
The RT-PCR reactions may be performed on a Perkin Elmer 480 thermocycler (Perkin Elmer Corp., Norwalk, CT, USA).

2.4 Regeneration and reuse of Dynabeads Oligo $(dT)_{25}$

The use of Dynabeads Oligo $(dT)_{25}$ allows for a one step hybridization since the oligo $(dT)_{25}$ is covalently attached to the Dynabead surface. The covalent binding makes it possible to regenerate the Dynabeads Oligo $(dT)_{25}$. The Dynabeads Oligo $(dT)_{25}$ may be reused a total of 4 times.

2.4.1 Protocol A - Regeneration using 0.1 M NaOH

This protocol is recommended if it is essential to avoid cross contamination between samples (19).

1. Resuspend the used Dynabeads Oligo $(dT)_{25}$ (250 µl) in **Reconditioning solution** (200 µl).
2. Transfer the suspension to a new RNase-free tube. Mix the content of the tube by shaking.
3. Incubate at 65°C for 2 min.
4. Place the tube in the magnetic concentrator for at least 30 s, and remove the supernatant. Repeat step 1, 2, and 4 twice.
5. Resuspend the Dynabeads in **Storage buffer Oligo $(dT)_{25}$** (200 µl). Mix briefly. Place the tube in the magnetic concentrator for at least 30 s, and remove and discard the supernatant.
6. Repeat this washing step 3 times or until the pH of the supernatant is below 8.0.
7. Resuspend the Dynabeads in the desired volume of **Storage buffer Oligo $(dT)_{25}$**. The Dynabeads are now reconditioned and ready for another mRNA isolation. Store the Dynabeads Oligo $(dT)_{25}$ at 4°C.

Note: Do not resuspend the Dynabeads in the original stock tube suspension stored in phosphate-buffered saline. No amplified product could be detected using Dynabeads Oligo $(dT)_{25}$ regenerated by protocol A. In subsequent extractions the quality of the RT-PCR product using regenerated Dynabeads, is comparable to that using fresh Dynabeads (Figure 2.5).

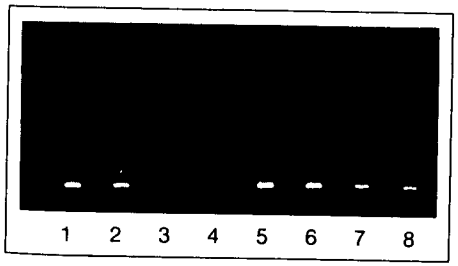

Figure 2.5
Evaluation of regeneration efficiency.
npRT-PCR on HIV RNA isolated from 250 µl serum containing 72 $TCID_{50}$ (50% Tissue Culture Infectious Dose) HIV-1, using *pol* specific primers. (72 $TCID_{50}$ HIV-1 is equivalent to approximately 100 to 1000 HIV RNA copies).
Regeneration efficiency Lane 1-4: Lane 1, 2: npRT-PCR of eluate after poly(A)$^+$ RNA isolation using Dynabeads Oligo $(dT)_{25}$. Lane 3, 4: npRT-PCR on regenerated Dynabeads Oligo $(dT)_{25}$ showing that no detectable RNA is left on the Dynabeads after regeneration with 0.1 M NaOH.
Capacity after regeneration Lane 5-8: Lane 5, 6: npRT-PCR of eluate after first poly(A)$^+$ RNA isolation using Dynabeads Oligo $(dT)_{25}$. Lane 7, 8: npRT-PCR of eluate after second poly(A)$^+$ RNA isolation using regenerated Dynabeads Oligo $(dT)_{25}$. Courtesy of A. Meijer, The Nat. Inst. of Publ. Health and Env. Prot., Bilthoven, The Netherlands.

2.4.2 Protocol B - Reuse for large scale mRNA isolation

This large scale protocol is recommended for repeat isolations from the same crude extract. The capacity of the Dynabeads Oligo $(dT)_{25}$ is 2 µg polyadenylated mRNA per mg Dynabeads. By using 1 ml (5 mg) of Dynabeads approximately 10 µg mRNA can be isolated. By reusing the Dynabeads on the same extract (without reconditioning of the Dynabeads) large amounts of mRNA can be isolated. Simply follow the mRNA isolation procedures in section 2.3. After

elution of the mRNA, the Dynabeads are washed once in **Lysis/binding buffer** before adding the crude extract and a new capture of mRNA is performed. This can be repeated several times until no mRNA is left in the extract (Figure 2.6).

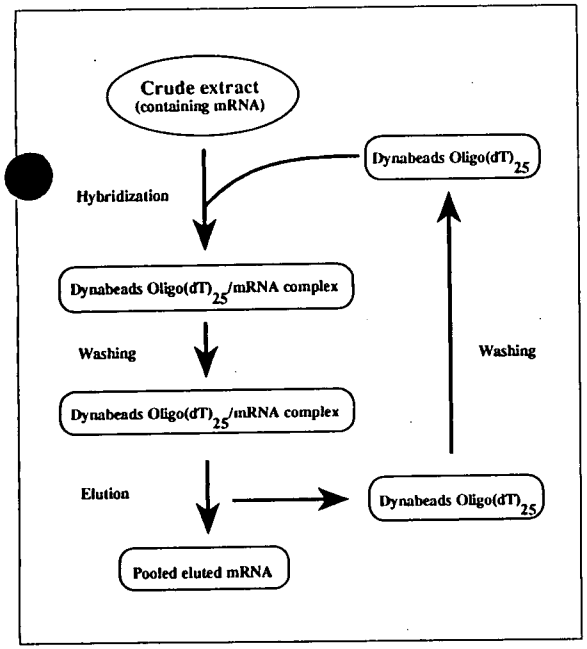

Figure 2.6 Schematic flow-chart for regeneration of Dynabeads Oligo $(dT)_{25}$ when performing large scale mRNA isolation.

2.5 Technical tips

Small and large scale direct mRNA isolation and suitable Dynal MPCs.

The use of Dynabeads Oligo $(dT)_{25}$ provide the highest purity mRNA directly from samples down to 1 cell (Figure 3.2). Thus allowing the detection of mRNA by RT-PCR from the early developmental stages of individual organisms or from highly specialized cells (e.g neurons).

Magnetic separation allows elution of target mRNA in very small volumes (down to 5 µl). Using a Magnetic Particle Concentrator like the Dynal MPC-M (optimum: 0.5-1.2 ml), Dynal MPC-E-1 (up to 0.5 ml), Dynal MPC-P-12 (up to 0.2 ml), Dynal MPC-9600 (up to 0.2 ml) or Dynal MPC-96 (up to 0.2 ml), the Dynabeads Oligo $(dT)_{25}$ can be used to isolate mRNA from multiple samples simultaneously. See also Table 2.4 on page 56.

The reusability characteristics of the Dynabeads Oligo $(dT)_{25}$ allow isolation of large amounts of mRNA directly from large samples of total RNA, cells or tissues (section 2.4.1 and 2.4.2). This is ideal for cDNA library construction from mRNA in solution, RNase A protection studies, Northern blot analysis especially of rare mRNA species, and *in vitro* translation studies.

For isolation of mRNA from large volumes of cells and tissues, Dynal recommends using a Dynal MPC-1 (up to 10 ml), Dynal MPC-6 (6 x up to 10 ml), Dynal MPC-2 (2 x 2-4 ml), Dynal

MPC-M (10 x 0.5-1.2 ml) or Dynal MPC-E (6 x up to 0.5 ml). This use of larger volumes will reduce the viscosity and improve the binding kinetics of the Dynabeads Oligo $(dT)_{25}$. See also Table 2.4 on page 56.

To estimate the amount of mRNA present in various starting materials the following data may be helpful as a guideline. A typical mammalian cell contains about 10^{-5} µg of total RNA, from which 1-5 % is mRNA. Typically, 50 to 75 µg of total RNA is isolated from 50 mg of muscle tissue and up to 400 µg is isolated from 50 mg of liver. The yield of total RNA from 10^7 cultured cells ranges from 50 to 80 µg for fibroblasts and lymphocytes and 100 to 120 µg for epithelial cells (20).

Preventing mRNA degradation by RNases

A: DEPC treatment

Diethyl pyrocarbonate treatment of water and salt solutions is important as the DEPC inactivates RNases by covalent modification. Wherever possible, the solutions should be treated with 0.1% DEPC for at least 1 hour at 37°C and then heated to 100°C for 15 minutes or autoclaved for 15 minutes to remove any traces of DEPC. Keep solutions for RNA work separate to avoid possible contamination of RNase.

Note: Buffers such as Tris cannot be DEPC treated as Tris react with DEPC. To prepare a Tris buffer, the water should be DEPC-treated and autoclaved before adding Tris. After addition of the Tris, the solution can (should) be autoclaved again.
DEPC is a suspected carcinogen and should be handled with great care.

B: Various technical tips for preventing mRNA degradation from RNases

1) A rapid lysis in **Lysis/binding buffer** is critical for obtaining undegraded mRNA. Thawing of frozen material prior to the lysis step must not occur. Work quickly and keep everything cold during preparation of starting material for the direct mRNA isolation protocol.
2) Use sterile disposable plasticware and rinse general laboratory glassware with chloroform.
3) Treat water, glassware and salt solutions with DEPC.
4) Wear disposable gloves and change them frequently.
5) RNase inhibitors may be added to the protocol at any step.
   Note: The addition of an RNase inhibitor is normally redundant. However, if storage of the eluted mRNA is required, addition of an RNase inhibitor at the elution step might be useful.
6) In general, remember to keep the eluted mRNA on ice to prevent mRNA degradation by RNases.

Viscosity reduction by DNA-shearing.

If your raw extract is noticeably viscous due to released DNA, a reduced viscosity is recommended to facilitate the magnetic separation and washing procedure. A DNA-shear step should be included in your protocol by either:

1) Passage through a syringe.
   For 1-8x10^6 cells in 1.0 ml **Lysis/binding buffer,** pressing approx. three times through a 21 gauge needle by a 1-2 ml syringe is recommended.
   Note: Use force to shear the DNA properly. In addition, be aware that repeated shearing causes foaming of the lysate due to detergent in the buffer. However, this should not effect the mRNA yield. The foaming can be reduced by a 30 s centrifugation.
2) Ultrasonication (21).

Elimination of possible rRNA contamination

In some cases trace amounts of ribosomal RNA has been observed in the mRNA samples. In applications like Northern blotting, trace amounts of rRNA contamination will not interfere with the analysis. For more critical applications like cDNA library constructions, a possible rRNA contamination should be avoided. The rRNA can be eliminated by reextracting the mRNA from the eluate. The Dynabeads may be reused for this double extraction if they are washed twice with **Washing buffer** before the second annealing of mRNA. If new Dynabeads are used we recommend that the Dynabeads are washed in 50 mM Na-pyrophosphate before the annealing of mRNA.

1. Follow the protocol for mRNA isolation from total RNA or directly from crude lysate.
2. [illegible]e the mRNA in **Elution buffer.**
3. Wash the Dynabeads twice in **Washing buffer** for reuse. New Dynabeads are washed in 50 mM Na-pyrophosphate in a Tris-buffer (pH 8) before use.
4. Four volumes of **Lysis/binding buffer** or **Binding buffer** is added to the mRNA sample and the annealing is done on a roller at room temperature for 3–5 minutes.
5. The beads are washed according to standard mRNA purification and the mRNA eluted in **Elution solution** for downstream applications. Solid-phase cDNA synthesis may be performed without elution, using the oligo $(dT)_{25}$ to prime the first cDNA strand. We recommend 2–3 extra washes in a RT-buffer to remove any SDS/LiDS before the reverse transcription.
   Note: An alternative to the reextraction protocol is to increase the stringency of the washing by lowering the salt concentration in the washing solution or performing the washes at 37°C.

Reuse of the Dynabeads Oligo $(dT)_{25}$

The Dynabeads Oligo $(dT)_{25}$ may be regenerated or washed for reuse four times (section 2.4). The reusability characteristics of the Dynabeads Oligo $(dT)_{25}$ allow isolation of large amounts of mRNA directly from large samples of total RNA, cells or tissues. This is ideal for cDNA library construction from solution phase mRNA, RNase A protection studies, Northern blot an[illegible]is especially of rare mRNA species, and *in vitro* translation studies.

A[illegible]RNA isolation from crude extracts of some kinds of tissue (e.g. rat pituitary) clumping of Dynabeads has been observed. In such cases we recommend trying one or both of the following steps to restore the Dynabeads to their original state.

1) Incubation with Proteinase K at 50°C for 1/2 h (removes protein contamination).
2) Wash three times with 70% isopropanol (removes lipid contaminants).

LiDS (SDS)

SDS is substituted by LiDS to prevent precipitation problems. Two extra washing steps without LiDS (SDS) should be included in the final washing.
Note: If the isolated poly A+ mRNA is to be used in enzymatic downstream applications, wash two extra times with **Washing buffer** (0.5 ml) or with the enzymatic buffer to be used. Changing to a new eppendorf tube prior to the final washing step will help eliminate contamination with SDS in the subsequent enzymatic downstream application.

Lysis in GTC

When lysis of desired tissue or cell sample is performed in a guanidine thiocyanate (GTC) based buffer, a dilution step is required to reduce the viscosity of the lysate. This will provide the proper conditions for efficient hybridization between the poly A^+ tail of the mRNA molecules and the oligo dT residue coupled to the surface of the Dynabeads. Further, sarcosyl should be used instead of SDS as the ionic detergent during the first washing steps if a GTC based mRNA isolation is performed. It has a higher solubility than SDS when using a highly chaotropic reagent such as GTC. The last washing steps should be performed without sarcosyl present. **Note:** GTC may be harmful by inhalation, ingestion and skin absorption. It may cause eye and skin irritation. GTC in contact with acid liberatestoxic gas.

Elution of mRNA in formamide for Northern analysis

For Northern blot analysis, the mRNA may be eluted directly in a loading buffer containing formamide and then loaded on the gel. For detection of rare mRNAs, between 0.5 and 3.0 µg of poly A^+ mRNA should be applied to each lane of the gel. **Note:** If your Northern blot gives a poor signal for the larger mRNAs (> 3kb), this may be explained by the capacity of the Dynabeads and the size distribution of the isolated mRNA. The smaller molecules tend to diffuse faster, and will bind preferentially to the Dynabead surface. Use more Dynabeads or reextract from the lysate, this should improve the yield of larger fragments.

Spectrophotometric determination of mRNA concentration and purity

The concentration of the mRNA may be determined by measuring the A_{260} of the final preparation. Measure the absorbance of the eluted mRNA at 260 nm, using cuvettes that have been soaked for 1 hour in concentrated HCl:Methanol (1:1) and then washed extensively in water which has been DEPC treated and autoclaved. A solution whose $A_{260} = 1$ corresponds to approximately 40 µg of mRNA/ml. The ratio between the readings at 260 nm and 280 nm gives an estimate of mRNA purity. An A_{260}/A_{280} absorbance ratio in the range 1.8 to 2.0 indicates a pure preparation.
Note: Make sure the solution is free of Dynabeads Oligo $(dT)_{25}$ as the Dynabeads will interfere with the spectrophotometric readings in an unfavourable way. If necessary, carry out another magnetic separation.

Agarose gel electrophoresis determination of mRNA quality

The quality of the isolated mRNA may also be checked by denaturing agarose gel electrophoresis. However, a rather large amount of mRNA is necessary to obtain a readable result. Northern blot determination of mRNA quality could be a more suitable tool for this purpose. Nevertheless, for mRNA quality determination using agarose gel electrophoresis, the mRNA should appear as a faint smear from approximately 10 kb down, depending on the tissue, with only trace rRNA bands. Note, that the presence of traces of rRNA should not affect the functionality of the isolated mRNA for most applications. For applications that require very high purity mRNA, please see the useful technical tips on how to limit rRNA contamination.

Table 2.4: Small and large scale **direct** mRNA isolations with suitable Dynal MPCs

Dynabeads Oligo ($dT)_{25}$ pr. mRNA isolation [ml]	Recommended hybridization volume of Lysis/ binding buffer [µl] pr. mRNA isolation	Number of mRNA isolations with 1 kit (without regeneration)	Number of mRNA isolations with 1 kit (with four regenerations)	Amount of animal tissue [mg] pr. mRNA isolation	Amount of cultured cells pr. mRNA isolation	Suitable Dynal MPC
1	3000 3 x 1000	1	5	80-200	4-16 x 10^6	Dynal MPC-6, Dynal MPC-1, Dynal MPC-2, Dynal MPC-M
0.5	2 x 1000	2	10	40-100	2-8 x 10^6	Dynal MPC-M or Dynal MPC-E
0.25	1000	4	20	20-50	1-4 x 10^6	Dynal MPC-M or Dynal MPC-E
0.2	750	5	25	16-40	1-3 x 10^6	Dynal MPC-M or Dynal MPC-E
0.15	500	6	30	12-30	1-2 x 10^6	Dynal MPC-M or Dynal MPC-E
0.10	333	10	50	8-20	1-1.5 x 10^6	Dynal MPC-E
0.075	250	13	65	6-15	1 x 10^6	Dynal MPC-E
0.03	100	30	150 (96)	3-8	5 x 10^5	Dynal MPC-E, Dynal MPC-P-12 Dynal MPC-9600, Dynal MPC-96

2.6 Buffers and solutions

Dynabeads mRNA DIRECT kit:

Phosphate-buffered saline (PBS) pH 7.4: The Dynabeads Oligo ($dT)_{25}$ are supplied as a suspension of 5 mg beads/ 1 ml PBS and 0.02% NaN_3.	137 mM NaCl 2.7 mM KCl 4.3 mM $Na_2HPO_4 \cdot 7H_2O$ 1.4 mM KH_2PO_4
Lysis/binding buffer (30 ml supplied with Dynabeads mRNA DIRECT kit)	100 mM Tris-HCl, pH 8.0 500 mM LiCl 10 mM EDTA, pH 8.0 1% LiDS 5 mM dithiothreitol (DTT)
Washing buffer with LiDS (SDS) (2 x 30 ml supplied with Dynabeads mRNA DIRECT kit)	10 mM Tris-HCl, pH 8.0 0.15 M LiCl 1 mM EDTA 0.1% LiDS
Washing buffer (30 ml supplied with Dynabeads mRNA DIRECT kit)	10 mM Tris-HCl pH 8.0 0.15 M LiCl 1 mM EDTA
Elution solution (1 ml supplied with Dynabeads mRNA DIRECT kit)	2 mM EDTA pH 8.0
Reconditioning solution (10 ml, supplied with Dynabeads mRNA DIRECT kit)	0.1 M NaOH
Storage buffer Oligo ($dT)_{25}$ (15 ml supplied with Dynabeads mRNA DIRECT kit)	250 mM Tris-HCl, pH 8.0 20 mM EDTA 0.1% Tween-20 0.02% Sodium azide

Dynabeads mRNA Purification kit:

Phosphate-buffered saline (PBS) pH 7.4: (1 ml, see above)	
2 x Binding buffer (2 ml supplied with Dynabeads mRNA Purification kit)	20 mM Tris-HCl (pH 7.5) 1.0 M LiCl 2 mM EDTA
Washing buffer (see above) (2 ml supplied with Dynabeads mRNA Purification Kit)	
Elution solution (see above) (2 ml supplied with Dynabeads mRNA Purification kit)	
Lysis buffer with NP-40 **(Not commercially available)**	1% NP-40 10 mM Tris-HCl (pH 7.5) 0.14 M NaCl 5 mM KCl

Note: All reagents used should be of analytical grade and RNase free.

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3. CONSTRUCTION OF IMMOBILIZED cDNA LIBRARIES FOR MULTIPLE RT-PCR AMPLIFICATIONS

3.1 General introduction

By using magnetic bead separation technology, pure, intact poly(A)$^+$ mRNA can be obtained either from total RNA preparations or directly from lysates of solid tissues and cell lines. An additional advantage of this method is that it is not necessary to elute off the captured mRNAs for subsequent construction of a cDNA library (1-4). It is in fact possible to produce solid-phase cDNA libraries specific for a particular cell type or tissue, directly on the Dynabead surface (1-11). The oligo(dT)-sequence bound to the bead surface is both used to capture the mRNA and as a primer for the reverse transcriptase to synthesize the first strand cDNA. This results in a covalently linked first strand cDNA library that can be used for specific applications like cDNA amplification (1-6), cDNA cloning (6) or subtractive hybridization (4, 7-10).

This protocol is adapted to the Dynabeads mRNA DIRECT protocol for mRNA isolation, but may be used in connection with the Dynabeads mRNA Purification Kit or other procedures for mRNA isolation using Dynabeads Oligo(dT)$_{25}$. Solid-phase synthesis requires that the Dynabeads with captured mRNA are washed properly before the enzymatic step, to remove detergent (LiDS) and salts. All reverse transcriptases tested were found to function in solid-phase

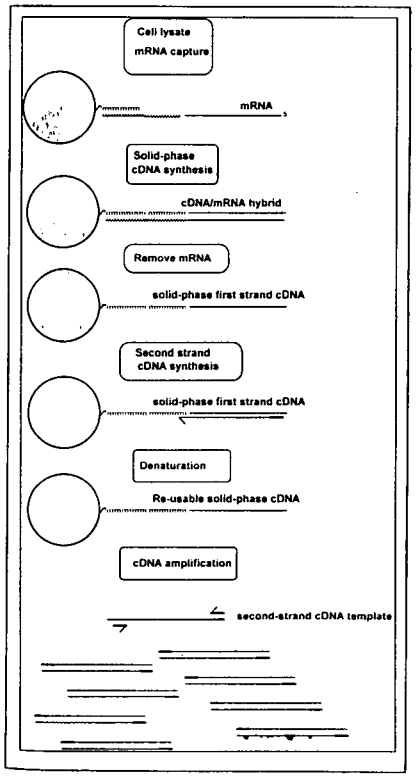


Figure 3.1 Reusable solid-phase cDNA library

cDNA synthesis (AMV, M-MLV, Superscript™, *rTth*, Retrotherm™), but we recommend using a thermostable enzyme like rTth or a combination of for example AMV and rTth.

The construction of a reusable solid-phase cDNA library allows multiple copies of the cDNA of a specific mRNA (second-strand cDNA) to be generated using a single-sided PCR with a specific primer (Figure 3.1). The second strand is melted off from the solid-phase template, the Dynabeads recovered by magnetic separation and the supernatant with the second strand cDNA used for amplification. The Dynabeads can be used for several specific second-strand cDNA syntheses.

Usually only small amounts of cDNA Dynabeads are necessary for cDNA amplification and there may be no need for reuse of these beads. It is then possible to move directly from a cDNA synthesis to a PCR amplification with the beads present during the cycling reactions. The solid-phase approach simplifies the identification and amplification of specific cDNA molecules for downstream analysis and applications.

RT-PCR has proven sensitive enough to detect transcripts from a few or even a single cell. However, these protocols typically require the lysis of cells in the presence of high concentrations of guanidinium thiocyanate and/or the purification of the RNA by caesium chloride ultracentrifugation and/or extraction and precipitation of the RNA. These steps are time-consuming and cumbersome and result in at least some loss of the RNA sample. By purifying mRNA with Dynabeads Oligo(dT)$_{25}$, RT-PCR has successfully been used to amplify transcripts from samples containing on the average less than 2 cells (Figure 3.2). The abundant transcripts of the β_2-microglobulin gene were isolated from the pre-B cell line Reh, and cDNA synthesis was done with the thermostable reverse transcriptase *rTth* (Perkin Elmer) for 15 minutes at 70°C. RT-PCR detection was possible both by cDNA synthesis on purified and eluted mRNA and on Dynabeads captured mRNA. The advantage of using Dynabeads on microscale mRNA isolations is that mRNA isolation and subsequent cDNA synthesis using a thermostable enzyme takes less than one hour, probably without significant loss of mRNA material.

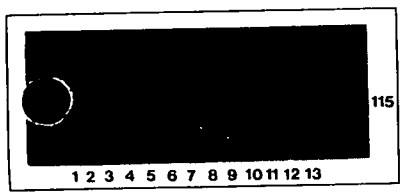


Figure 3.2 Example of RT-PCR using minute amounts of sample. Solid-phase cDNA synthesis using mRNA isolated from Reh-cells (pre-B cell line) and PCR with β_2-microglobulin specific primers. Lane 1: 100 cells with RNase treatment prior to RT-PCR; lane 2: 100 cells; lane 3: negative control; lanes 4–13: mRNA from on the average 1.8 cells. Only lane 9 is negative. The probability of getting one negative reaction is less than 0.02 if single cells are not detectable. Courtesy of A. Deggerdal, The Norwegian Radium Hospital, Norway.

RACE PCR (rapid amplification of cDNA ends) has proven to be a valuable tool for obtaining the ends of gene transcripts. Lee and Vacquier (5) made a solid-phase cDNA library for RACE by dG tailing of the cDNAs. 5' RACE was done by amplification with a 3' gene specific primer and a 5' adaptor poly(dC) primer. For the 3' end amplification a 5' end specific primer and a 3' poly(dT) primer were used. The amplicons are separated from the cDNA Dynabeads using a MPC, and the recovered dG-tailed cDNA Dynabeads can be reused for at least five rounds of PCR (5). By using one biotinylated primer in the amplification, the amplicons can subsequently be sequenced by standard solid-phase protocol as described in chapter 1.

Construction of a cDNA library from tiny amounts of mRNA by PCR-amplification of cDNA that has been tailed at the 3' end of the first strand cDNA has been a successful strategy. By synthesizing an immobilized cDNA library on Dynabeads all manipulations can be carried out in one tube and drawbacks of other protocols like yield loss due to multiple transfers and precipitations can be overcome. Lambert and Williamson (6) were able to clone a representative library from about 5 ng polyadenylated RNA from tomato root tips. After cDNA synthesis unprimed oligo(dT)$_{25}$ on the Dynabeads were removed by T4 DNA polymerase before the cDNA strands were A-tailed by terminal transferase. The second cDNA strands were synthesized with an oligo(dT) primer with a tail sequence. The second strand cDNAs were released and amplified and subsequently cloned.

3.2 Materials required

- Dynabeads Oligo (dT)$_{25}$
- Washing buffer with LiDS (SDS)
- Washing buffer
- RT Buffer
- RT-mix with nucleotides
- Reverse transcriptase (AMV, M-MLV, Superscript, *rTth*, Retrotherm™)
- Elution Solution
- TB Buffer
- Magnetic Particle Concentrator (Dynal MPC, see appendix B)
- Water bath
- Programmable heat-block (DNA Thermal Cycler 480 or GeneAmp® PCR System 9600, Perkin Elmer Corp., Norwalk, CT, USA)
- Sterile, RNase-free microtubes of Eppendorf type
- Sterile, RNase-free pipettes and pipette tips
- Deionized, RNase-free water
- Diethyl pyrocarbonate (DEPC)

Note: Buffers and solutions are described in section 3.5.

Table 3.1 Recommended volumes and magnets for small to large scale cDNA synthesis.

	Small scale	Medium	Large scale
Dynabeads Oligo(dT)$_{25}$	10 µl = 50 µg	60 µl = 300 µg	250 µl = 1250 µg
Cells	up to 150000	up to 1 million	up to 4 million
Recommended no. of cells	80000-100000	500000-750000	2-3 million
Max. volume mRNA isolation	250 µl	500 µl	1 ml
Max. volume cDNA synthesis	20 µl	50 µl	200 µl
Recommended tube	Microtube/Microamp	Microtube/Eppendorf	Eppendorf
Recommended magnets	Dynal MPC-P-12 Dynal MPC-9600	Dynal MPC-P-12 Dynal MPC-E	Dynal MPC-E Dynal MPC-M

3.3 Protocols

3.3.1 Synthesis of a solid-phase cDNA library

1. Capture mRNA with Dynabeads Oligo(dT)$_{25}$ either directly from cell lysates or from total RNA preparations as previously described (chapter 2). Titer the number of cells and the amount of Dynabeads used. The rule of thumb is 10 µl beads [50 µg] to about 100000 cells when working with proliferating cells. The numbers given are for medium scale libraries, numbers for small scale given in square brackets. The mRNA is isolated as described in the Dynabeads mRNA DIRECT kit protocol. The mRNA/Dynabead complexes are washed twice with Washing buffer with LiDS and once with Washing buffer as described in chapter [illegible] but the mRNA is not eluted from the Dynabeads.
2. To completely remove the LiDS and LiCl, the 300 µg Dynabeads [50 µg] with the captured mRNA are washed three times in 250 µl cold RT-buffer [50 µl]. The Dynabeads are thoroughly resuspended each time and transferred to a new RNase-free tube between washing step 2 and 3.
3. The RT-buffer is removed after the third washing step and the Dynabeads are resuspended in RT-mix with nucleotides, buffer, RNase inhibitor and enzyme. A maximum of 300 µg Dynabeads [50 µg] are used per cDNA synthesis in a volume of 50 µl [20 µl].
4. The cDNA synthesis is performed as recommended by the manufacturer of the RT. For AMV usually at 42°C and for M-MLV or Superscript at 37°C, for one hour. The cDNA synthesis using a thermostable enzyme (*rTth*) is performed for 5 min at 50°C and 10 min at 72°C. A combination of enzymes may be used; cDNA synthesis at 37–42°C for 45 min, a rapid change of buffer by applying the magnet, and incubation at 72°C for 10 min with the thermostable enzyme.

   Note: The synthesis can be done using a heating block, a water bath or a thermocycler. If possible, the tubes should be mixed every 10 min to keep the Dynabeads in suspension to improve the cDNA synthesis. We recommend to use a hybridization oven to obtain constant rolling and optimal cDNA synthesis.
5. After the first-strand cDNA synthesis is completed, the beads are collected with a magnet and the RT-mix removed. The beads are resuspended in 50 µl of TE or Elution solution and heated to 95°C for 1 min. The beads are immediately collected by using a magnet, and the supernatant containing the melted mRNA is removed and discarded.
6. [illegible]e cDNA Dynabeads are washed once in TB-buffer and stored in this buffer at 4°C. It is [illegible]so possible to store the solid-phase cDNA library in TE, SSC or 70% ethanol. The single-stranded cDNA library is stable in this form in nuclease-free solutions.

3.3.2 Amplification of genes from a reusable cDNA library immobilized on Dynabeads.

1. Remove the storage solution from the solid-phase cDNA library and resuspend the beads in PCR-buffer. Up to 100 µg cDNA Dynabeads are used per PCR-amplification (we recommend 20–50 µg). Aliquot the beads, apply the magnet and remove the PCR-buffer. Add PCR-mix with primers and *Taq* polymerase, and resuspend the cDNA Dynabeads properly.
2. Cycle once to make the 2nd strand cDNA with a 5 min extension at 72°C. Melt the strands at 94°C for 2 min, place on magnet and transfer the supernatant with the second-strand cDNA to a new PCR tube. Continue the cycling reaction at standard extension at 72°C (1–2 min). Wash the Dynabeads, and resuspend them in storage solution for reuse.

   Note: If it is not necessary to reuse the beads, just run the PCR with the beads present through the cycling reactions as described in 3.3.3.

3. Amplified PCR products are analysed by agarose electrophoresis.

   Note: Subsequent solid-phase sequencing of the amplified cDNA is possible if one biotinylated primer and one non-biotinylated primer is used in the PCR reaction. First, remove any Dynabeads Oligo(dT)$_{25}$. Perform a binding of the biotinylated PCR-product to Dynabeads Streptavidin M-280. Proceed with making a single-stranded sequencing template as described in chapter 1.

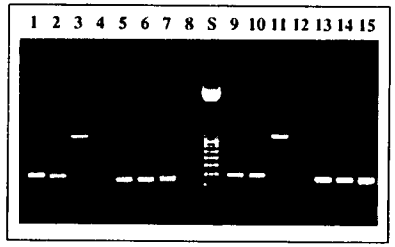


Figure 3.3 RT-PCR using immobilized cDNA on Dynabeads as template.
Direct mRNA isolation from 250000 cultured HL-60 cells with 100 µg Dynabeads Oligo(dT)$_{25}$ per RT-PCR reaction, lane 1, 2, 4, 5 and double purification of mRNA lane 9, 10, 12, 13. Solid-phase cDNA synthesis with rTth polymerase for 5 min at 50°C and 10 min at 72°C. One third of the PCR products were analysed by agarose gel electrophoresis. Lane S shows a 100 bp ladder. Lane 1–4 and 9–12 show amplification with primers for the human MECL-1 gene (12) and lane 5–7 and 13–15 amplification of the human glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). Lane 3, 7, 11 and 15 are amplification of genomic DNA as controls with a larger genomic product for the MECL-1 gene; lane 4 and 12 are negative controls without reverse transcriptase added (no DNA contamination) and lane 8 is the negative PCR control. These results show little difference between direct isolation and double purification of mRNA using Dynabeads. No DNA contamination was detected. GAPDH primers; forward 5'-ACAGTCCATGCCATCACTGCC-3', reverse 5'-GCCTGCTTCACCACCTTCTTG-3', amplicon 228 bp. MECL-1 primers; forward 5'TGGCGGTGCTAGAAGACC-3', reverse 5'-GCTTACTCCACCTCCATAGCC-3'. MECL-1 cDNA amplicon 304 bp, genomic amplicon 893 bp. Cycling program: 94°C for 5 min, 34 cycles of 1 min 61°C, 1 min 72°C, 20 sec 94°C. (K. Lycke and F. Larsen, Dynal R & D).

3.3.3 Amplification of genes from an immobilized cDNA library with Dynabeads present in the PCR.

1. Remove the storage solution from the solid-phase cDNA library and resuspend the beads in PCR-buffer. Up to 100 µg cDNA Dynabeads are used per PCR-amplification (we recommend 20–50 µg). Aliquot the beads, apply the magnet and remove the PCR-buffer. Add PCR-mix with primers and *Taq* polymerase and resuspend the cDNA Dynabeads properly.
2. Perform the PCR-amplification with the beads present.

   Note: Even cDNA Dynabeads used in a full PCR cycling program can be reused. Just keep in mind that the Dynabeads will be contaminated with the amplicon. The reuse should be to amplify a different transcript.

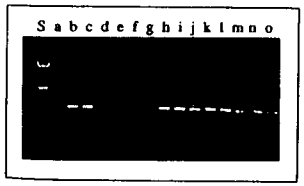


Figure 3.4 PCR-amplification is not inhibited by Dynabeads.
Direct isolation of mRNA from 300000 cultured HL-60 cells with 100 µg Dynabeads Oligo(dT)$_{25}$. Solid-phase cDNA synthesis with rTth polymerase for 5 min at 50°C and 10 min at 72°C. Amplification of the glyceraldehyde-3-phosphate-dehydrogenase gene in a 50 µl PCR volume with different amounts of cDNA immobilized on Dynabeads. Lane S with a 100 bp ladder, negative PCR control in lane a and positive control without beads in lane b and c. 200 µg Dynabeads (d-e); 150 µg (f-g); 100 µg (h-i); 50 µg (j-k); 25 µg and 10 µg (n-o). No inhibition is seen when 100 mg or less is used. Primers and PCR are described in Fig. 3.3. (K. Lycke and F. Larsen, Dynal R & D)

3.3.4 One tube RT-PCR with *rTth* polymerase

The solid-phase technology using Dynabeads is compatible with Perkin Elmer's EZ *rTth* RNA PCR kit. Included in this kit are reagents to perform the reverse transcription of RNA to cDNA and the subsequent PCR-amplification, all in one tube. The combination of direct mRNA isolation with Dynabeads and one tube RT-PCR provides a very fast and powerful technique. This protocol is a rapid and reliable method for screening numerous samples for specific cellular or viral RNA appearance or disappearance. The RNA to be transcribed and later amplified can be as long as 3 kb, although 100 to 1000 bases are more typical and easier to amplify. When high fidelity conditions are required, we recommend the use of separate cDNA synthesis and amplification reactions.

1. Capture mRNA with Dynabeads Oligo(dT)$_{25}$ either directly from cell lysates or from total RNA preparations as previously described. Titer the number of cells and the amount of Dynabeads used. The rule of thumb is 10 µl beads (50 µg) to about 100000 cells when working with proliferating cells. We recommend using 20–50 µg of Dynabeads per RT-PCR reaction with mRNA from up to 150000 cells. The mRNA is isolated as described in the Dynabeads mRNA DIRECT kit protocol. The mRNA/Dynabead complexes are washed twice with Washing buffer with LiDS and once with Washing buffer as described in chapter 2, but the mRNA is not eluted from the beads.
2. To completely remove the LiDS and LiCl, the 50 µg Dynabeads with the captured mRNA are washed three times in 50 µl cold RT-buffer. The Dynabeads are thoroughly resuspended each time and transferred to a new RNase-free tube between washing step 2 and 3.
3. The mRNA/Dynabead complexes are resuspended in 50 µl EZ buffer with *rTth* polymerase, nucleotides, Mn^{2+} and primers. Both the cDNA synthesis and the amplification are performed using a thermocycler. The cDNA synthesis is performed for 5 min at 50°C and 25 min at 61°C. This is immediately followed by the cycling reactions to amplify transcripts.

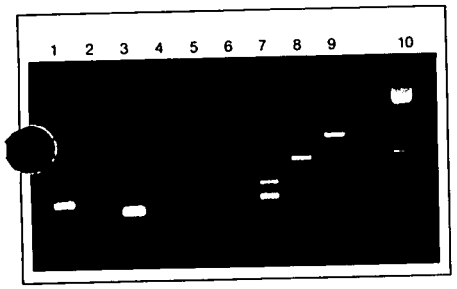
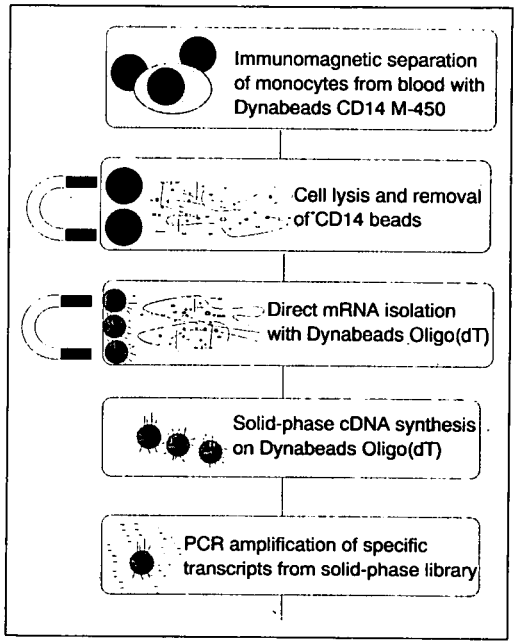


Figure 3.5 One-tube RT-PCR using Dynabeads. Direct isolation of mRNA from 150000 cultured Daudi cells with 50 µg Dynabeads Oligo(dT)$_{25}$ per lane. Solid-phase cDNA synthesis with rTth polymerase for 5 min at 50°C and 25 min at 61°C and 34 amplification cycles with the same polymerase in a 50 µl volume. Amplification of different transcripts for the GAPDH gene (lane 1), CD19 gene (lane 2) and the protein kinase PSK-H1 gene (lane 3), (12). Lane 4–6 are negative control without rTth but else as lane 1–3. Lane 7–9 are positive controls with amplification of genomic DNA with the three sets of primers. In lane 1 and 7 an extra primerset (HUC) is included to detect genomic DNA as shown by the upper band in lane 7. Lane 10 is a 100 bp ladder. PSK-H1 primers; forward 5'-AACCGTACCCGGCTGTACCG-3', reverse 5'-AGAGGCAGCCATGCTCACCA-3', 183 bp cDNA amplicon. CD 19 primers; forward 5'-GCGGATGGAGCCTTGGGGTC-3', reverse 5'-GTTGCTTCCCGGCTGGGGTC-3', 320 bp cDNA amplicon and 722 bp genomic amplicon. HUC primers for human genomic DNA, chromosome 16; 5'-TGAAAGCCTGGTGCCTTTGTTAGCC-3' and 5'-CCTCGACCCAGACCGAAATACCCAG-3', HUC-amplicon 347 bp. GAPDH primers and cycling program as described in Fig. 3.3. (K. Lycke and F. Larsen, Dynal R & D).

3.3.5 Immunomagnetic separation of monocytes and lymphocytes combined with direct mRNA isolation and RT-PCR

A very powerful approach to gene regulation studies is the combination of immunomagnetic cell separation and bead-assisted mRNA isolation and RT-PCR. This enables a very rapid isolation of specific cells (see chapter 2 and 7), and the cells can be lysed while attached to the Dynabeads (Figure 3.6). This has been proven to work for monocytes (Figure 3.6) and T- and B-lymphocytes (Figure 3.7), and the procedure could easily be modified for other cell types.



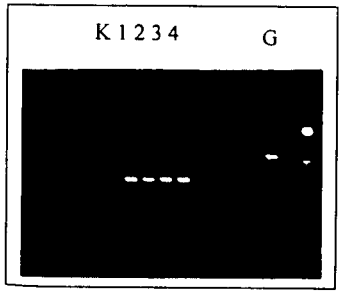


Figure 3.6 Direct mRNA isolation from monocytes was done as described in protocol 2.4.3 with Dynabeads M-450 CD14. cDNA synthesis was done as described in protocol 3.3.1 with rTth polymerase. Amplification was done on the human MECL-1 gene (12) with 34 cycles. Agarose gel electrophoresis of one third of the PCR products. Lane K is the negative cDNA synthesis control and lane 5 the negative PCR control. Primers and cycling program as in Fig. 3.3. Lane 1 to 4 is amplification of transcripts from different amounts of cells; monocytes from 50 µl blood in lane 1, 80 µl in lane 2, 125 µl lane 3 and 160 µl in lane 4. Lane G shows the positive PCR control using genomic DNA as template. The size of this amplicon is 893 bp compared to the 304 bp cDNA amplicon.

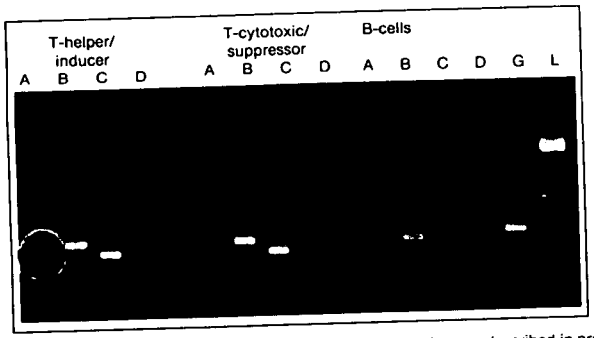


Figure 3.7 Direct mRNA isolation from lymphocytes was done as described in protocol 2.3.4 with Dynabeads M-450 CD8 for T cytotoxic/suppressor cells, Dynabeads M-450 CD4 for T-helper/inducer cells and Dynabeads M-450 CD19 for B cells. cDNA synthesis was done as described in protocol 3.3.4 with rTth polymerase. Amplification was done on the human GAPDH and PSK-H1 genes(12) with 34 cycles as described in Fig. 3.3. Agarose gel electrophoresis of one third of the PCR products. The A lanes show controls of genomic DNA contamination with HUC primers, whereas the D-lanes are negative controls for cDNA synthesis where no reverse transcriptase has been added, but *Taq* polymerase added for the PCR. The B lanes show amplification of the GAPDH gene (228 bp) and the C lanes show amplification of PSK-H1 gene. The 183 bp amplicon of the latter gene is only possible to amplify from cDNA. Lane G is a positive control of genomic DNA for HUC primers and lane L a 100 bp ladder. Primers and amplification as described in Fig. 3.3 and Fig. 3.5. (K. Lycke and F. Larsen, Dynal R & D).

3.4 Technical tips

DNA contamination

Using a direct mRNA isolation method there is a risk for DNA contamination. This is usually no problem with the mRNA DIRECT system, but may occur when large amount of material or difficult tissues like spleen is used. We generally recommend to start with a maximum of 1 million cells in a 1 ml isolation volume (Lysis/binding buffer) to isolate pure mRNA for cDNA synthesis. It is important to reduce the viscosity either by diluting the sample or by DNA-shearing as described in section 2.5. Another option is to purify the mRNA twice with Dynabeads Oligo$(dT)_{25}$. There are several possibilities for controls to ensure the detection of cDNA and not genomic DNA;

a) Choose the primers in neighbouring exons to obtain different amplicon sizes for cDNA and genomic DNA as shown for MECL-1 in Figure 3.3 and 3.6, PSK-H1 and CD19 in Figure 3.5.

b) Include a negative control with no reverse transcriptase as shown in Figure 3.7.

c) Use genomic DNA specific primers to detect any contamination, like HUC primers used in Figure 3.5 and 3.7.

d) Use RNase-free DNase to treat the mRNA sample before cDNA synthesis to get rid of any DNA contamination.

3.5 Buffers

RT-buffer (M-MLV, AMV, Superscript™, *rTth*, Retrotherm™)	10 mM Tris-HCl, pH 8.3 75 mM KCl
RT-MIX rTth	10 mM Tris-HCl pH 8.3 90 mM KCl 1 mM $MnCl_2$ 0.2 mM each dGTP, dATP, dTTP, dCTP 20 Units RNase inhibitor 10 Units/50 ml
RT-MIX M-MLV/ Superscript™	50 mM Tris-HCl, pH 8.3 75 mM KCl 10 mM DTT 3 mM $MgCl_2$ 0.5 mM each dGTP, dATP, dTTP, dCTP 20 Units RNase inhibitor 200 Units enzyme/20 ml
RT-MIX AMV	50 mM Tris-HCl, pH 8.3 50 mM KCl 1 mM DTT 10 mM $MgCl_2$ 0.5 mM each dGTP, dATP, dTTP, dCTP 20 Units RNase inhibitor 20 Units enzyme/20 ml
RT-MIX Retrotherm	10 mM Tris-HCl pH 8.3 50 mM KCl 1.5 mM $MgCl_2$ 0.75 mM $MnSO_4$ 0.2 mM each dGTP, dATP, dTTP, dCTP 20 Units RNase inhibitor 5 Units/50 ml
TE Buffer	10 mM Tris HCl, pH 7.5 1 mM EDTA
1x Elution solution	2 mM EDTA, pH 8.0
1x TB Buffer	20 mM Tris-HCl, pH 8.4 50 mM KCl 1.5 mM $MgCl_2$ 0.1 mg/ml BSA

EZ-rTth mix: as described in the Perkin Elmer kit

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4. SUBTRACTIVE HYBRIDIZATION

4.1 General introduction

Subtractive hybridization methods are used to isolate genes that are specifically expressed in one cell type or a specific tissue. This includes genes that are differentially regulated during activation or differentiation of cells in addition to genes that are involved in illnesses like cancer and virus infection. Several different strategies have been developed to achieve this goal (1-6). They are all based on subtracting away the common transcripts between different cell types or tissues, leaving the specific transcripts (mRNA) for further analysis.

Traditionally, subtraction protocols have involved hybridization of mRNA from one cell type (target) to first-strand cDNA from another cell type (subtractor) and subsequent hydroxyapatite chromatography to remove hybrids; screening of cDNA libraries from one cell type with cDNA probes from a different type of cell or subtractive hybridizations using biotinylated probes and avidin resins. Although successful to varying degrees, these systems often require large amounts of poly(A)$^+$ mRNA or highly purified single-stranded DNA, and they are technically demanding and labour intensive.

A major improvement to the subtraction approach has been the use of magnetic bead assisted subtraction (7-12). This solid-phase approach provides a fast and reliable way of generating subtracted probes or subtracted cDNA libraries. One other main advantage of this method is that the subtractor Dynabeads easily can be regenerated and used for at least three different subtractions, each involving three hybridization steps (11, 12). The use of magnetic separation technology permits easy manipulations and buffer changes for optimal reactions. We recommend to use Dynabeads Oligo(dT)$_{25}$ for subtractive hybridization, but the combination of biotinylated oligo(dT) and Dynabeads M-280 Streptavidin can also be used. Both types of

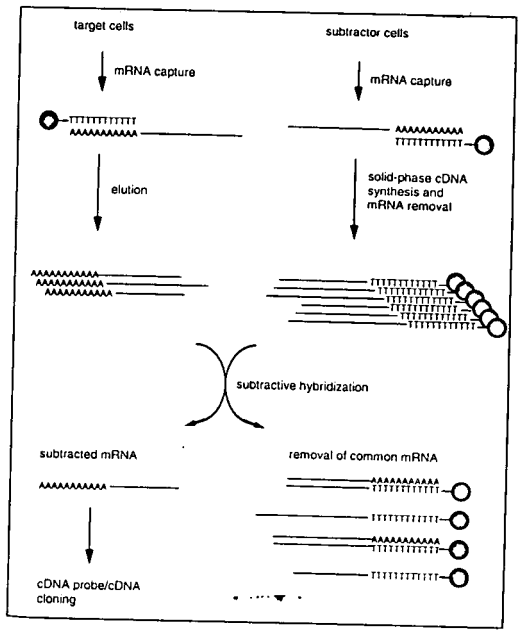

Figure 4.1 Subtractive hybridization using immobilized cDNAs on Dynabeads (I). The subtractive hybridization strategy used by Sharma *et al.* (10) and Aasheim *et al.* (11). The approach is to hybridize mRNA from a target cell-type with first-strand cDNA from a subtractor cell-type, immobilized on Dynabeads (10, 11). The subtracted mRNA is left in the supernatant after removal of the cDNA Dynabeads with the captured mRNA (common mRNA population). The subtracted mRNA is reverse transcribed to radiolabeled cDNA and used as a probe to screen existing cDNA libraries (11) or both first and second strand cDNA are synthesized for cDNA cloning (10).

Dynabeads show low non-specific binding of nucleic acids and proteins, but the advantage of the former is that the oligo(dT) is covalently linked to the Dynabead surface.

The main principles of using magnetic Dynabeads are outlined in Figure 4.1. mRNA from the subtractor tissue or cell population is isolated using Dynabeads Oligo(dT)$_{25}$ and directly converted to the complementary 1. strand cDNA, leading to immobilized cDNA on Dynabeads as described in chapter 3. mRNA from the target tissue/cell population is isolated with Dynabeads Oligo(dT)$_{25}$ and eluted from the beads, solved in a hybridization buffer, heated to 70°C and mixed with subtractor cDNA immobilized on Dynabeads (10-12). The mRNA is hybridized to the subtractor cDNA at 65-68°C for 20-24 hours (11), and common transcripts are removed by collecting the beads with the cDNA/mRNA hybrids. Two more hybridizations are recommended to give optimal subtraction. The same subtractor cDNA Dynabeads can be used again for regeneration by eluting the captured mRNA. After the final hybridization step the specific mRNA for the target cells is left in the solution. The remaining mRNA can be captured with unused Dynabeads, and used to generate a subtraction cDNA library or to make a radioactive cDNA probe to screen existing cloned cDNA libraries. The specificity of the subtraction procedure can be confirmed by Northern blot analysis.

Another approach is to make immobilized cDNA libraries on Dynabeads from both the target mRNA population and the subtractor mRNA (Figure 4.2). The second strand cDNA is synthesized by random priming of the target cDNA. The second strand fragments are eluted and mixed with an excess of immobilized subtractor cDNA. The common fragments are allowed to anneal and are removed by magnetic separation. The unique fragments left in the supernatant are used as a probe to screen a cDNA library (7-9). This method was used to create a probe to screen a retina cDNA library. The probe identified several retina-specific genes like rhodopsin, green visual pigment and interphotoreceptor retinoid-binding protein (7).

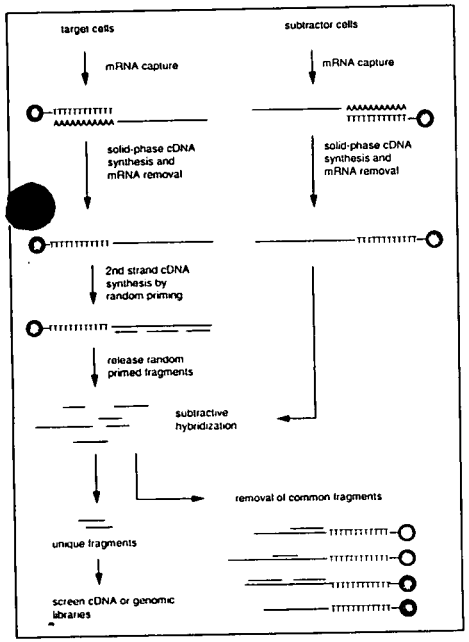

Figure 4.2 Subtractive hybridization using immobilized cDNAs on Dynabeads (II).
The subtractive hybridization strategy as used by Rodriguez and Chader (7). The principle is to make immobilized cDNA libraries on Dynabeads from both the target mRNA population and the subtractor mRNA. The second strand cDNA is synthesized by random priming of the target cDNA. The second strand fragments are eluted and mixed with an excess of immobilized subtractor cDNA. The common fragments are allowed to anneal, and then removed by magnetic attraction. The unique fragments left in the supernatant are used as a probe to screen a cDNA library (7-9).

One of the problems with subtraction techniques is that specific mRNAs/cDNAs are subtracted away by common sequences (e.g. gene families or repetitive sequences). Rodriguez *et al.* (7) reduced this problem by using short, random primed fragments from target mRNAs. However, by performing the hybridization at high stringency only perfectly matched hybrids will be subtracted away as shown by Aasheim *et al.* (11). This gives an end-result not only of incomplete cDNA-probes, but cell-specific mRNAs ready for cloning. Alternatively, the mRNAs can be used to make immobilized cDNA-library on Dynabeads.

4.2 Materials required

- Dynabeads Oligo (dT)$_{25}$
- Magnetic Particle Concentrator - Dynal MPC (see Appendix B)
- 6x SSPE Buffer
- 6x SSC Buffer
- 0.1% SDS
- 0.15 M NaOH containing 0.1 M NaCl
- 2.5 mM EDTA
- Reverse transcriptase
- Test tubes, glassware, pipettes

4.3 Protocols

In this section two subtraction methods are presented. Both methods have been shown to give excellent subtraction results (7-12). The mRNA used can be isolated either directly from crude cell lysates or from total RNA as described in chapter 2. The immobilized cDNA is synthesized on Dynabeads as described in chapter 3. To create a full-length cDNA library we recommend to use a combination of modified M-MLV reverse transcriptase like Superscript II (BRL) and a thermostable reverse transcriptase like rTth (Perkin Elmer). The mRNA isolation and cDNA synthesis is just outlined in this section, whereas a more detailed description is given for the generation of the subtractor cDNA Dynabeads, followed by the subtractive hybridization and the generation of a subtracted probe. One must be aware that Dynabeads with bound mRNA or immobilized cDNA should be handled carefully, in particular when resuspending the material. We recommend to use siliconized microcentrifuge tubes for the subtractive hybridization and for the generation of a subtractive probe, to avoid non-specific binding of nucleic acids to plastics, and thereby loss of precious material.

4.3.1 Method I

This method is essentially as described by Sharma *et al.* (10) and Aasheim *et al.* (11) and the principle is shown in Figure 4.1. It is possible to scale up the protocol to obtain more material for the generation of a probe or a library. This can be important when the difference between the subtractor and the target mRNA population is small. The protocol is described for hybridization in 4.5 x SSPE with 0,1% SDS at 65-68°C, but the stringency can be increased by reducing the salt concentration to 1 X SSPE.

Preparation of immobilized subtractor cDNA:

1. mRNA from the subtractor cells (10 μg) corresponding to about 20-50 million cultured cells is isolated with Dynabeads Oligo(dT)$_{25}$ as described in chapter 2. Since the capacity of these Dynabeads are about 2 μg mRNA per mg, the required amount of Dynabeads is about 5 mg or 1 ml. After mRNA capture and purification, the mRNA/Dynabeads complexes are washed 2-3 times with a reverse transcriptase buffer to remove all traces of LiCl and detergent which otherwise will inhibit the enzymatic reactions.

2. cDNA is synthesized onto the Dynabeads as previously described in chapter 3. The immobilized cDNA is synthesized by using the oligo(dT) as primer and f. ex. 2000 U Superscript II (BRL) in the recommended buffer (200 µl). The reaction mixture is incubate at 37°C for 60 min. We recommend to use a hybridization oven to obtain constant rolling and optimal cDNA synthesis. The Dynabeads are collected with a magnet (Dynal MPC) and resuspended in an appropriate buffer with 50 U rTth reverse polymerase and incubated at 70°C for 10 min. The last step is done to extend the cDNA strands further by opening up any secondary structures in the mRNA. The use of rTth increases the efficiency of the cDNA synthesis by 60–80% as measured by incorporation of radioactivity (12).
3. After cDNA synthesis, the Dynabeads are resuspended in 2 mM EDTA and the mRNA is [illegible]ted away by heating at 95°C for 3 min followed by immediate magnetic separation of [illegible]e beads and removal of the solution. (The eluted mRNA can be controlled by performing an electrophoretic separation in a denaturing agarose gel.) The subtractor cDNA Dynabeads are washed carefully three times in 0.5 ml TE pH 8.0. The subtractor beads can be stored in TE at 4°C for several months. With an estimated 25% efficiency of the 1. strand cDNA synthesis reaction, approximately 2.5 µg immobilized cDNA is generated from 10 µg mRNA (12).
4. **mRNA isolation from the target cell population:**
About 0.3 to 0.6 µg mRNA is isolated from 1–2 million target cells with 500 µg or 100 µl Dynabeads Oligo(dT). This results in approximately a ten fold excess of subtractor over the target mRNA to be subtracted. Perform the direct mRNA isolation in a 1 ml volume and include DNA shearing if necessary (chapter 2) to avoid any contamination of DNA since the latter is also likely to result in a RNase contamination. The purified mRNA is eluted and transferred to the hybridization buffer (4.5 X SSPE and 0,1% SDS). Alternatively, the mRNA can be prepared in larger amounts and stored at -70°C in the hybridization solution.

Subtractive hybridization:

5. The subtraction is carried out in a microcentrifuge tube. Both the subtractor cDNA Dynabeads in TE buffer and the target mRNA in hybridization buffer are in parallel heated to 68°C for 3 min. The buffer is removed from the subtractor beads before the beads are combined with the hybridization solution (200 µl) with mRNA. The cap of the microcentrifuge tube is closed and wrapped in parafilm to prohibit evaporation, and the tube is incubated in a hybridization oven at 65-68°C for 20–24 hours under constant rolling to achieve [illegible]roper mixing of subtractor beads with mRNA.
6. After the first hybridization, the subtractor Dynabeads with the mRNA/cDNA hybrids are collected with a magnet (MPC-E) and the hybridization solution is transferred to a new RNase free tube and stored on ice for the second hybridization. The subtractor cDNA beads are regenerated by adding 20 µl water followed by 3 min heating at 68°C and removal of the water with mRNA. (The eluted mRNA can be controlled by performing an electrophoretic separation in a denaturing agarose gel.) The subtractor beads are resuspended in 100 µl TE.
7. Step 5 and 6 are repeated twice to perform in total three rounds of subtractive hybridization. Before the last hybridization, 250 ng of yeast tRNA is added to the hybridization solution. This is done to reduce/avoid any loss of the small amount of specific mRNA left, by non-specific binding to Dynabeads or plastic.
8. After the third subtractive hybridization, the subtractor Dynabeads are removed and the hybridization solution will contain specific mRNA from the target cells. The mRNA is isolated from the solution by adding 20 µl or 100 µg Dynabeads Oligo(dT), prewashed in hybridization buffer. Capturing of the mRNA is done by incubation for 5–10 min at room temperature under constant rolling. The mRNA/Dynabeads complexes are washed once with Washing buffer with LiDS and twice with Washing buffer as described in chapter 2.

9A. **Generation of a subtracted probe** can be done in several ways from the subtracted mRNA. Lambert (13) has described an approach that amplifies the mRNA by RT-PCR followed by radioactive labeling of the product. Alternatively, the direct labeling can be done incorporating radioactive nucleotides in the cDNA synthesis. This requires more mRNA than the former method, which may be difficult to obtain when the difference between the subtractor and the target mRNA population is small.

When using the PCR based approach to generate a probe, the subtracted material can be checked for enrichment. One tenth of the material can be used to make a Southern blot and hybridized with a probe for a "housekeeping" gene (e.g. common messages). If no signal is obtained in the subtracted material compared to the starting material, the subtraction can be considered to be optimal.

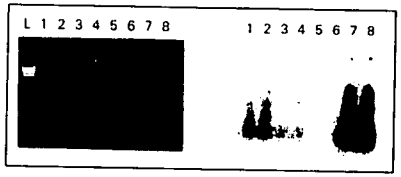

Fig. 4.3 **Evaluation of the subtractive hybridization.** PCR amplified subtracted material (lane 1-6) and amplified starting material or target mRNA population (lane 7-8) were separated by agarose electrophoresis (left picture) and Southern blotted onto a nylon membrane. This membrane was hybridized with a probe for actin (right picture). The highly abundant transcripts for the "housekeeping" actin gene is the reason for the strong signals seen in lane 7 and 8 with the non-subtracted material. Whereas lane 5-6 show no signals which indicates an optimal subtraction. The cells used in this experiment is Reh, a pre-B cell (target) and Daudi (subtractor). Lane S is Hind III digested phage lambda-DNA, which shows that the amplified material is in the size range of 500 bp to 6-7 kb. Courtesy of Hans-Christian Aasheim, The Norwegian Radium Hospital.

9B: **Construction of immobilized subtracted cDNA library on Dynabeads** is a good alternative when working with limited amounts of material (see chapter 3). This cDNA can be used for multiple PCR amplifications and stored for several months. One approach is to use degenerate primers against conserved regions from gene families for amplification. This has been used to amplify novel protein kinases (12).

4.3.2 Method II

This method is essentially as described by Rodriguez and Chader (7) and the principle is shown in Figure 4.2.

Preparation of immobilized subtractor and target cDNA:
mRNA is isolated both from the target and the subtractor tissue/cells and converted to immobilized cDNA on Dynabeads. Double amount of mRNA and Dynabeads are used for generating the subtractor cDNA.

1. The polyadenylated mRNA is captured on Dynabeads Oligo(dT)$_{25}$ and washed properly (see chapter 2 and 3) before cDNA synthesis. For example, 50-100 µg of total RNA is incubated with 250 µg Dynabeads Oligo(dT)$_{25}$ to capture the polyadenylated mRNA. To get a representative mRNA population captured on Dynabeads, one should avoid to use too much mRNA sample since the short transcripts will anneal faster to the beads than the long mRNAs.
2. The immobilized cDNA is synthesized using the oligo(dT) as primer and by using f. ex. 200 U Superscript II (BRL) in the recommended buffer, with incubation at 37°C for 60 min. We recommend to use a hybridization oven to obtain constant rolling and optimal cDNA synthesis. The Dynabeads are collected with a magnet (MPC) and resuspended in an appropriate buffer with 20 U rTth reverse polymerase and incubated at 70°C for 10 min. The last step is done to extend the cDNA strands further by opening up any secondary structures in the mRNA. By combining any M-MLV enzyme and the thermostable Retrotherm (Epicentre) the same buffer can be used in both incubations (7).

3. The mRNA is melted away by heating in TE or water at 95°C for 2 min. The Dynabeads with immobilized cDNA are collected with a magnet (MPC) and resuspended in 50 µl TE.

Preparation of labeled secondary cDNA from target:

4. An amount of Dynabeads with 100 ng immobilized cDNA from the target is copied and labeled by random priming using 200 mCi of ^{32}P dCTP. After incubation the random priming mix with unincorporated nucleotides is removed from the Dynabeads by applying a magnet.

5. The labeled secondary cDNA strands are eluted from the cDNA Dynabeads either in 100 µl TE by heating to 95°C for 2 min or by addition of 100 µl 0.15 N NaOH and incubation at room temperature for 5 min. In both cases, 100 µl 10X SSC is added with 2 µg of carrier RNA.

Subtractive hybridization:

6. The labeled target cDNA strands in 5X SSC are added to subtractor cDNA immobilized on Dynabeads (prepared from the subtractor mRNA population as described in step 1 to 3). The cDNA strands generated from 100 ng target cDNA is added to 200 ng immobilized subtractor cDNA on Dynabeads. The mix is denatured at 94°C for 2 min and hybridized at 55°C for 60 min with constant rolling in a hybridization oven.

Subtracted probe for screening:

7. The cDNA probes that fail to anneal to the immobilized subtractor cDNA is used to screen a cloned cDNA library from the target cells. A more specific probe can be generated by repeating the subtraction step. The same subtractor Dynabeads can be used after removing the captured target fragments as described in step 5.

4.3.3 Generation of an unlimited supply of a subtracted probe

One drawback of the methods described is that the amount of the material remaining in solution after the subtractive hybridization may be insufficient for screening purposes. In addition, there may not be enough material for several rounds of subtraction to be performed. This may be true when the amount of material (mRNA) is limited or when the two sources of mRNA are very similar (target/subtractor). Coche *et al.* (9) have made a method to solve this problem. The main feature of the method is to reanneal the subtracted cDNA fragments (Method II) back onto the immobilized subtractor cDNA, followed by extension and restriction of the newly synthesized double-stranded cDNAs. Linkers are ligated to the resulting blunt ended molecules which can then be amplified by PCR.

4.4 Buffers and solutions

TE Buffer	10 mM Tris HCl, pH 7.5 1 mM EDTA
Hybridization buffer	4.5 X SSPE 0,1% SDS
6X SSPE	500 mM NaCl 33 mM $NaH_2PO_4xH_2O$ 3.3 mM EDTA

Dissolve the reagents in 800 ml of H_2O. Adjust the pH to 7.4 with NaOH. Adjust the volume to 1 l with H_2O. Sterilise by autoclaving.

6X SSC	0.9 M NaCl 0.09 M sodium citrate

Dissolve the reagents in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 10 M solution of NaOH. Adjust the volume to 1 l with H_2O. Sterilise by autoclaving.

Combined buffer for M-MLV, Superscript and Retrotherm reverse transcriptase:	10 mM Tris-HCl pH 8,3 50 mM KCl 1,5 mM $MgCl_2$ 0,75 mM $MnCl_2$ 10 mM dNTP (2,5 mM each) 20 Units RNase inhibitor 200 Units Superscript II (BRL) 5 Units Retrotherm (Epicentre)

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5. DNA AND RNA HYBRIDIZATION

5.1 General introduction

Dynabeads M-280 Streptavidin and Dynabeads Oligo(dT)$_{25}$ provide an efficient solid phase alternative to nitrocellulose and nylon membranes for hybridization capture of specific target DNA/RNA molecules. Biotinylated nucleic acids immobilized on Dynabeads M-280 Streptavidin may be hybridized to complementary DNA/RNA sequences. The large binding capacity and excellent reaction kinetics of the beads provide high efficiency and low background. A practical example of this strategy is given in chapter 6, "Direct purification of M13 single-stranded DNA templates for DNA sequencing". Subtractive hybridization using immobilized cDNA on Dynabeads to remove hybrids from the solution, is described in chapter 4.

When compared to conventional library screening methods (i.e. *in situ* filter hybridization), the Dynabeads technology has several advantages. Target DNA or RNA sequences from complex libraries are directly captured in solution and purified. Magnetic separation of target

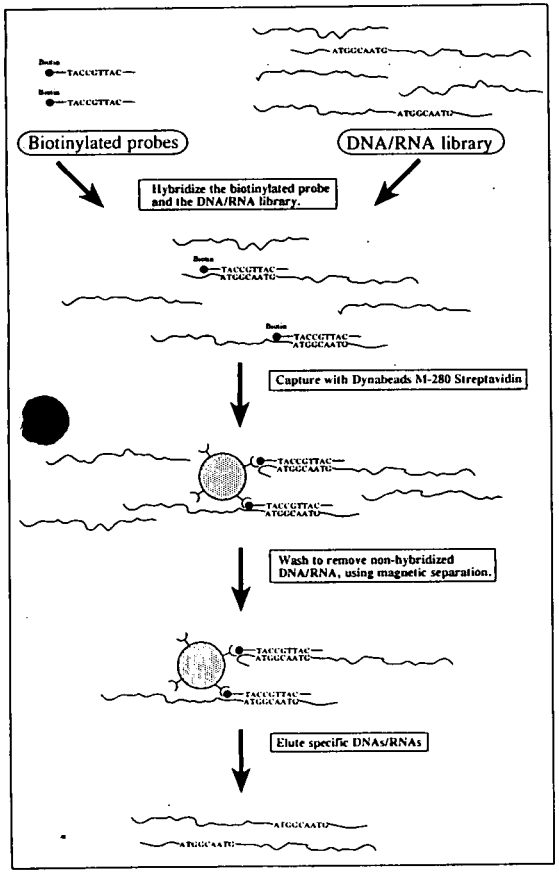

Figure 5.1
A schematic drawing of the basic concept for hybidization/isolation of nucleic acids using Dynabeads.

sequences increases productivity and reduces reagent costs by eliminating filter preparation, centrifugation and precipitation steps. The use of nylon and nitrocellulose membranes in complex hybridization reactions often results in low efficiency and high background. The Dynabeads rapidly isolate highly enriched target DNA or RNA sequences directly from complex libraries with minimal background.

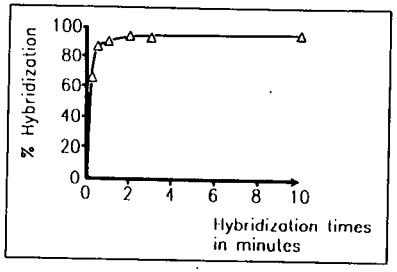

Figure 5.2 Hybridization kinetics of Dynabeads with oligo(dT)$_{25}$ probes to oligonucleotides. Ten picomoles of oligo(dA)$_{25}$ and 100 µg oligo(dT)$_{25}$ beads in 50 µl hybridization solution was used in each experiment. Hybridization times tested were 0.25, 0.5, 1.0, 2.0, 3.0 and 10.0 minutes. Control beads (Dynabeads M-280 Streptavidin) with no oligo(dT)$_{25}$ attached gave less than 0.001% hybridization after 3 minutes (1).

5.2 Materials required

- Dynabeads M-280 Streptavidin/Dynabeads Oligo (dT)$_{25}$
- Magnetic Particle Concentrator, Dynal MPC (see Appendix B)
- Biotinylated probe (when using Dynabeads M-280 Streptavidin)
- Binding and Washing buffer
- 6x SSPE Buffer
- 6x SSC Buffer
- 10% SDS
- 0.15 M NaOH containing 0.1 M NaCl
- Test tubes, glassware, pipettes
- Reverse transcriptase, Taq polymerase
- Cot1 DNA
- PCR primers
- G50 spin columns
- Hybridization solution

5.3 Direct hybrid capture with immobilized probes

Biotinylated DNA or RNA probes are bound to the Dynabeads through the high affinity biotin-streptavidin linkage ($K_d = 10^{-15}$ M). The beads with immobilized DNA or RNA are incubated with a sample containing target DNA or RNA sequences. Upon hybridization, the captured target sequences are separated from the heterogeneous suspension using a magnet (Dynal MPC). To elute isolated target DNA or RNA sequences, standard elution procedures are used such as change in pH, ionic strength or temperature.

This direct hybrid capture method has been successfully used for detection of enterovirus RNA (2, 3), Hepatitis C RNA (4, 5) and Hepatitis B virus DNA (6), for isolation of poly(A)$^+$ RNA (1), ssM13 DNA (7) and different mitochondrial tRNAs (8, 9), and for quantification of c-myc PCR products (10).

5.4 Indirect hybrid capture

Biotinylated DNA or RNA probes are allowed to hybridize with target DNA or RNA sequences in solution. Dynabeads are then added to capture hybrids containing the biotinylated DNA or RNA. Magnetic separation and elution of the target DNA or RNA sequences is completed in minutes. For complex hybridization reactions or isolation of low abundance DNA or RNA sequences, Dynal recommends the indirect method.

This indirect hybrid capture method has been successfully used for selective isolation of expressed cDNAs within large genomic regions cloned in cosmids and YACs (11-23). This is

thoroughly discussed as a reference application in section 5.6. Other applications for this method include detection of PCR-amplified HIV-1 DNA (24), quantification of specific mRNA (25) and quantification of PCR products (26).

The direct selection of cDNAs by magnetic capture utilises biotin-streptavidin Dynabead technology, thereby allowing a rapid and reproducible isolation of cDNAs encoded within large genomic regions (11-23). Biotinylated cloned genomic DNA is hybridized in solution with amplifiable cDNA (Fig. 5.3). The biotinylated DNA and the attached cDNAs are captured on Dynabeads M-280 Streptavidin and the non-specific hybrids are dissociated by stringent washing. The cDNAs are eluted, amplified, cloned and analysed as region-specific sublibraries of cDNA from certain tissue(s). The technique results in an enrichment of the selected cDNAs between 10^3 to 10^6 (15). It allows the analysis of several large genomic intervals of varying complexities and can be applied to the isolation of expressed sequences from various tissues in parallel (21, 22).

The pool of enriched cDNA that is PCR-amplified, is either used as probe to screen conventional cDNA libraries; or to isolate pools of transcripts to make a region-specific library; or as probe for isolating YACs or cosmids. This technique is limited when the transcription pattern of the gene of interest is not known. In such cases, cDNA from different tissues and developmental stages are required (15). A second serious problem inherent in this method is the co-selection of pseudogenes. This can be transcribed pseudogenes, but more likely non-transcribed pseudogenes that select cDNAs which belong to genes located somewhere else in the genome. However, this problem can be sorted out by sequencing.

An essential requirement for the analysis of genomes is the identification of functionally important sequence elements which are often evolutionary conserved. Unfortunately, not all these potentially informative sequences are transcribed. For this reason, a variation of the protocols for magnetic capture of cDNAs has been described (19). This is a procedure for selective isolation of conserved sequences, which is based on hybridization of PCR-amplifiable DNA fragments from the whole genome of one species to biotinylated DNA from a genomic region of another species. The interspecies hybrids are immobilized on Dynabeads M-280 Streptavidin. The captured DNA fragments are eluted, amplified and cloned. This method generates sublibraries of conserved interspecies sequences.

5.5 Triplex affinity capture (TAC)

Triple-helix formation has proven to be a powerful and fairly general approach to DNA targeting. It is based upon the specific binding of pyrimidine oligonucleotides to a polypurine strand in duplex DNA, forming a local triple-helical structure (28 and references therein). Studies have demonstrated the potential of triple-helix mediated capture for enrichment and screening of recombinant DNA libraries (29-31).

TAC approach: Biotinylated oligonucleotide probes are immobilized onto the Dynabeads M-280 Streptavidin. Double-stranded target DNA is captured via triple helix formation to the biotinylated probe on the beads. Target DNA is separated with a Dynal MPC and eluted through a change in pH.

Recently, biotinylated oligonucleotides were demonstrated to form tight triple-helix complexes with supercoiled plasmid DNA for the purpose of purifying plasmids from cell lysates (32). In a follow up, this approach was used to separate cosmid insert DNA molecules from the cosmid vector DNA (28). This is a simple and rapid method for isolating pure insert DNA (> 95%) which can be used to generate random fragments for shotgun sequencing. In this way, time and money can be saved by avoiding sequencing vector subclones. However, the library needs to be generated in a specially designed cosmid vector (28).

Another approach for targeting of linear DNA duplexes has been developed by Sena and Zarling*(27). Stable four-stranded hybrids are formed by addition of two short complementary RecA protein-coated DNA probes homologous to sequences in the target. The RecA proteins

are removed without destroying the hybrids and these can be isolated and detected by affinity capture on Dynabeads M-280 Streptavidin. This approach provides a means of isolating any desired gene or chromosomal DNA fragment.

5.6 Reference application: Rapid selection of cDNAs encoded within large genomic regions in clones.

A major problem in "gene hunting" using positional cloning and candidate gene approaches is the screening of large genomic regions for coding sequences. Many techniques have been developed in the past to address this problem. Most of these techniques do have a number of difficulties, such as low efficiency or low signal-to-noise ratio (direct cDNA screening using YACs), or they are technically very complex (exon trapping). For a review of these techniques, see Parrish and Nelson (11).

The identification and isolation of human transcripts as cDNA clones can be accompanied by a second problem due to cDNA abundance or sequence complexity (15). Only a few percent of the total genomic DNA is transcribed into mRNA and only 10-20% of all mRNAs can be expressed in any differentiated cell type (about 10000 genes per cell type). The level of expression of these genes may be from 200000 mRNA molecules per cell to less than one mRNA per cell on average. Approximately 30% of the genes are expressed at less than 10 copies per cell at any given time of cellular development. The search is further complicated when using a complex tissue as a source for cDNA, as this will decrease the representation of cell-type specific mRNAs further. Recently, a number of approaches have been suggested to overcome these problems by combining the specificity of hybridization, the sensitivity of PCR amplification and the power of magnetic capture and separation.

Hybridization in solution
The scheme of selection or enrichment of cDNAs encoded within large genomic regions is given in Figure 5.3. The original protocols for cDNA selection, required immobilization of genomic clones on nylon membranes, followed by hybridization of this filter-bound DNA with cDNA in solution. The magnetic bead methods maintain both hybridization partners in solution, which allows a better control of the hybridization conditions compared to the filter-based methods. In the former protocols, the genomic clones were biotinylated and captured after hybridization by Dynabeads M-280 Streptavidin. For information on how to biotinylate DNA see Appendix E. Other solid supports for capturing hybrids (streptavidin-agarose, biotin-cellulose), resulted in considerably higher non-specific binding. The strength and stability of the biotin-streptavidin coupling allows DNA manipulations at any desired stringency, denaturation and elution of annealed cDNAs or simple and efficient change of buffers. Therefore, when using Dynabeads a very flexible system for blocking repetitive sequences, hybridization, washing and elution is generated.

Source of genomic DNA
The genomic DNA used for cDNA selection can be cloned in any genomic DNA cloning system (lambda phages, cosmids, P1 phages or YACs). All genomic sources result in an enrichment of cDNAs encoded by the insert. However, the enrichment with cosmid or P1 DNA is more efficient than lambda clones due to better insert-to-vector ratio. Also YAC DNA is not as favourable as cosmid-DNA due to the problems of generating pure YAC DNA. However, selection artefacts working with YAC clones can be overcome by pre-blocking the YAC DNA with total Yeast DNA (15). By doing this, only a 2-fold efficiency in favour of the cosmid clones is obtained (15).

Source of cDNA
Any source of cDNA can be used in a selection experiment, as long as it is PCR amplifiable. Oligo(dT) primed cDNA libraries, Mbo I digested library of this type (16), a normalised short insert library, random primed cDNA library (13) or a combination of random and oligo(dT)

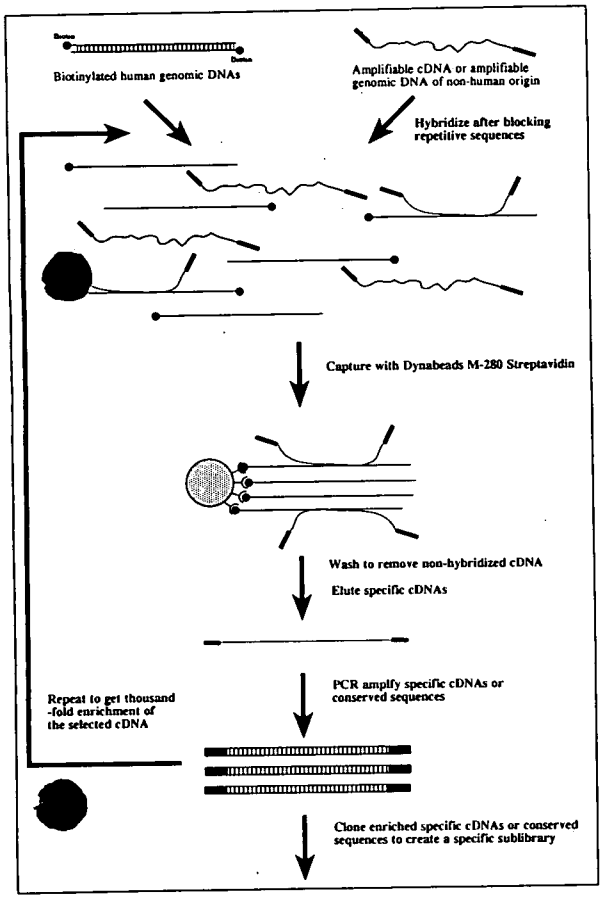

Figure 5.3 The scheme of selection or enrichment of cDNA encoded within large genomic regions.

primed library (14) have been reported thus far. There are pros and cons for each method, but normalised libraries, as well as random primed libraries, are the cDNA source of choice, with the disadvantage being the intermediate cloning step. In addition, care should be taken to avoid DNA contamination in the isolated RNA. Dynal recommend to use Dynabeads Oligo $(dT)_{25}$ to isolate mRNA free of contaminants (chapter 2), to use this mRNA to make the cDNA for example as an immobilized library (chapter 3) for making short cDNA fragments by random priming and amplification (13).

Blocking repetitive sequences
The main problem of cDNA selection is to prevent repetitive sequences present in cDNA and genomic clones to hybridize with each other. This is commonly done by pre-annealing either one or both of the sources with various other nucleic acids. Total human DNA or human cot1 DNA have been used. The current protocol recommended uses cot1 DNA to pre-anneal repetitive sequences of the genomic source or the cDNA source (13). An unsolved problem so far is the suppression of low copy repeats or repeats that are only present in certain sub-chromosomal regions (20-22).

Hybridization and elution
Various buffers have been described for the protocol, including formamide/standard salt phosphate EDTA (SSPE), formamide/SSC (14), SSC/SDS (13). Various mass ratios of cDNA to genomic DNA have been used, ranging from 10000-fold excess of cDNA to equal amounts of both sources. All magnetic capture procedures described so far use Dynabeads M-280 Streptavidin to bind the biotinylated genomic DNA. The immobilized DNA with hybrids are washed at high stringency and the specific cDNA fragments are eluted either at high temperature or with high pH. The eluted cDNAs are subjected to PCR amplification, with the conditions dependent on the primers used. The resulting material is either cycled back for a second or third round of selection and amplification using fresh genomic material, or is cloned directly into phage or plasmid vectors for further analysis.

5.6.1 Results obtained with direct cDNA selection using Dynabeads

Abe (12): cDNAs encoding novel germ cell specific genes were identified by a direct selection method applied on genomic clones from mouse chromosome 17.

Forster and Rabbitts (13): Identification of genes involved in translocation breakpoints (see the presentation and protocol in section 5.7)

Korn *et al.* (14, 15, 18): The technique was applied to the analysis of transcripts from two cosmid contigs, together encompassing a region of 900 kb in chromosome Xq28. This resulted in the identification of 81 cDNA clones. A transcriptional map covering a 300 kb region identified seven new genes.

Morgan *et al.* (16): Direct cDNA selection was applied to a 425 kb YAC with the IL-4 and IL-5 genes. After two selection rounds, 24 cDNAs were evaluated. Nine cDNAs encoded the interferon regulatory factor 1 gene, six clones identified a gene homologous to a murine P600 gene, which is coexpressed with the interleukin genes. The nine remaining cDNAs were unique and non redundant. As much as a 100000 fold enrichment was obtained.

Peterson *et al.* (17): Direct cDNA selection from a 1.2 Mb region surrounding the marker D21S55 on chromosome 2. Two rounds of hybrid selection and amplification. 76 of 120 cDNA clones were easily shown to be derived from this region.

Tagle *et al.* (20-22): A 2.2 Mb interval at chromosome 4p16.3 was analysed for the presence of transcribed sequences. After two rounds of enrichment using inserts from a fetal brain cDNA library, specific transcripts found in low abundance were enriched several 1000-fold with an associated decrease in non-specific, ubiquitous transcripts such as β-actin.

Wevrick *et al.* (23): Isolation of a novel gene from the Prader - Willi syndrome smallest region of deletion overlap, on human chromosome 15q. A 325 kb YAC clone was used to isolate expressed sequences by indirect selection capture with Dynabeads.

5.7 Protocol

This is a procedure for rapid isolation of cDNAs corresponding to genes within a YAC clone mainly as described by Forster and Rabbitts (13). Random cDNA fragments are hybridized to PCR-generated biotinylated fragments of total DNA from a yeast strain harbouring a YAC clone. The hybrids can be captured by Dynabeads to facilitate subsequent cloning of the cDNA molecules. The cDNA fragments have common PCR linkers. The outline of the procedure is shown in Figure 5.4. The essential components are that whole yeast DNA is used to select cDNA molecules as hybrids, that the hybrids are captured with Dynabeads, and that these cDNA species are subsequently amplified by PCR prior to cloning. Total yeast cellular DNA harbouring an appropriate YAC clone is converted to random, biotinylated fragments by PCR amplification with a redundant oligonucleotide primer. These fragments are hybridized to cDNA prepared from RNA which is derived from a suitable source (i.e. one that would be expected to express the gene of interest). This cDNA is prepared using a different redundant primer which is added to both ends of each molecule and which carries a sequence allowing

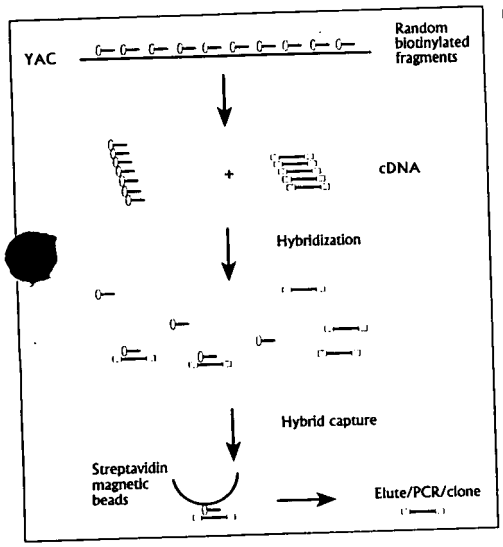

Figure 5.4 Diagrammatic scheme of direct selection procedure using Dynabeads (13). Total yeast DNA containing a YAC of interest is converted to random biotinylated fragments by PCR amplification and hybridized to cDNA which is made with PCR priming sites on both ends. The hybrids are selected with Dynabeads M-280 Streptavidin and the captured cDNA eluted, amplified by PCR and cloned.

subsequent amplification. After hybridization of the cDNA with cot1 DNA, to mask the repetitive sequences within cDNA molecules, the cDNA is hybridized to the biotinylated yeast DNA. Hybrids are captured and recovered with streptavidin-coated magnetic Dynabeads. The cDNA is simply eluted from the beads by heating, and amplified by PCR and cloned. Since the random priming procedure gives a size distribution of the cDNA population on average about 300 bp, repetitive regions and unique regions are mainly on separate DNA fragments.

The model system used to illustrate the protocol was the cloning of cDNAs encompassing the translocation breakpoint t(4;11)(q21;q23) which occur in subsets of acute leukaemia (13). The junction of t(4;11) has been determined to be a fusion of the *MLL* gene from chromosome 11 band q23 with the *AF4* gene on chromosome 4. The YAC clone used, consists of about 250 kb human DNA originating from chromosome 11 band q23 with the *MLL* gene and at least one other gene *p941*. cDNA was prepared from the RS411 cell line carrying the t(4;11) translocation. Two rounds of cDNA selection were carried out and the resulting cDNA cloned into a plasmid vector. The clones were hybridized with probes for the two genes. The selection represent at least a 1000 fold enrichment for the first round and a subsequent further enrichment (Table 1). The size of the selected clones ranged from 200 to 500 bp. Two clones were sequenced and showed to contain the breakpoint with sequences both for the *MLL* and the *AP4* genes.

Table 5.1 cDNA enrichment by selection

Gene	unselected	1. selection	2 selections
MLL	0.001%	2%	7.3%
p941	?	0%	0.8%

Biotinylated YAC DNA-fragments are made in 100 µl PCR reaction (standard buffer) using 0,5 µg YAC DNA (YAC = total yeast DNA), 0,5 µg primer C, A, G, T (equal mixture, 0,125 µg each). The mix was denatured for 10 min at 94°C. Cycling was; 1 min at 94°C, 1.5 min at 30°C and 3 min at 42°C for 5 cycles. This reaction was split into ten new 100 µl PCR reactions with 10 µl Biomeg1 primer (100 ng/µl). Cycling program; 1 min at 94°C, 1 min at 62°C and 5 min at 72°C for 35 cycles with a final 10 min synthesis at 72°C. These were passed through a G50 spin column before the eluates were pooled and boiled for 5 min to denature the amplicons.
Primer C: 5'-GATATTACCCTGTTATCCCTANNNNNC-3'
Primer A, G and T are the same except for the substitution of A, G and T respectively as the 3' terminal base instead of C.
Biomeg1: 5'-biotin-GATATTACCCTGTTATCCCTA-3'

cDNA fragments were made using 100–300 ng mRNA or 5 µg total RNA in 20 µl reverse transcriptase reaction in RT-Buffer with 0.5 µg primer-mix N6CAS, for 1 hour at 42°C. At the end of the first strand cDNA synthesis, the reaction was diluted to 100 µl with water and 10 µl transferred to a 100 µl PCR-reaction with 1 µg primer mix N6CAS. The PCR reaction was denatured for 10 min at 94°C and cycled 5 times for 1 min at 94°C, 1.5 min at 30°C and 3 min at 42°C. Four new 100 µl PCR reactions were set up each with 10 µl of the N6CAS PCR reaction and 1 µg CAS primer. Amplification was for 35 cycles with 1 min at 94°C, 1 min at 62°C and 5 min at 72°C. Each reaction was passed through a G50 spin column and eluates pooled.
N6CAS: 5'-GATGCGGCCGCCTCGAGCTCANNNNNN-3'
CAS: 5'-GATGCGGCCGCCTCGAGCTCA-3'

Blocking of repetitive sequences in the cDNA fragments was done by adding 5 µg Cot I DNA (Gibco) and the mixture precipitated by addition of 1/10 vol 2M NaAC pH 5 and 2 vols EtOH. After 16 hours at - 20°C, the precipitate was collected, washed with 70% EtOH, dried *in vacuo* and dissolved in the hybridization mix (2x SSC, 0,1% SDS and 1x Denhardt's mix). This was overlaid with mineral oil and boiled for 5 min and hybridized for 4 hours at 68°C. After this blocking step, the oil was removed and the hybridization mix was diluted with 250 µl water and passed through G50 spin columns in 100 µl fractions and pooled.

cDNA selection by hybridization: The boiled, biotinylated YAC DNA (the product of 10 x 100 µl PCR reactions) was added to the preblocked cDNA (do not boil at this stage) and the nucleic recovered by EtOH precipitation over night as previously described. The precipitate was spun down, and the pellet washed in 70% EtOH and dried *in vacuo*. Selection hybridization was carried out after dissolving the precipitate in 50 µl hybridization mix with 6X SSC, 0,1% SDS and 1X Denhardt's mix. The mix was overlaid with mineral oil and hybridized at 68°C for 16 hours.

Hybrid capture: Nucleic acids were passed through G50 spin column, and the flow-through adjusted to 1 ml Binding and Washing buffer (B & W). 100 µl Dynabeads M-280 Streptavidin (1 mg) were pre-washed three times in 0,5 ml B & W, added to the nucleic acids and suspended by rotation for 30 min at room temperature. The Dynabeads with captured hybrids were collected with a magnet (MPC) for 1 min and washed twice at room temperature with 0,5 ml Hybrid wash (0,1 x SSC, 0.1 % SDS) followed by three times 15 min at 65°C with 0,5 ml Hybrid wash. Finally the captured cDNAs were eluted with 50 µl water for 10 min at 80°C.

Repeated cDNA selection: For second and further rounds of selection, biotinylated YAC DNA was prepared as above and the blocking hybridization with cot1 DNA was omitted. 4 x 100 µl PCR reactions with CAS primer were set up with 10 µl each of the selected and eluted cDNA. cDNA selection, hybrid capture and cDNA elution were carried out as described above.

Amplification of selected cDNA: The final eluted cDNA was amplified using 10 µl of the eluate in a 100 µl PCR reaction with CAS primer. This primer has a site for the restriction enzyme Not I, allowing cloning into plasmid vectors such as pBSpt (Stratagene).

5.8 General hybridization conditions

From a steric point of view, hybridization to nucleic acids bound to Dynabeads is intermediary between filter hybridization and hybridization in solution. This does not, however, significantly affect the melting temperature of DNA/DNA or DNA/RNA hybrids. Parameters such as hybridization time, salt concentration, and hybridization temperature can therefore be determined

as for filter hybridization (33-35). A sound discussion of hybridization parameters can be found in Hames and Higgins (35). Several computer programmes for the determination of melting temperatures of oligonucleotides are commercially available. All common hybridization conditions can be used with the Dynabeads M-280 Streptavidin. The following guidelines are recommended for DNA/RNA hybridization protocols. For further technical information on general nucleic acid hybridization condition, see (33-35).

Hybridization based capture with Dynabeads M-280 Streptavidin can be performed according to one of two methods:

Direct hybrid capture: A biotinylated probe is bound to the beads before the hybridization is started. In this case, the beads are present throughout the hybridization.

Indirect hybrid capture A biotinylated probe is used in the hybridization. After annealing to the probe, the hybrids are immobilized on Dynabeads M-280 Streptavidin.

For complex hybridizations, Dynal recommends the indirect method, for hybrid capture.

Hybridization buffers To maximize the rate of annealing, hybridizations should be done in buffers such as:

- For a typical oligonucleotide probe: 6 X SSPE/SSC
- For a probe larger than 200 bp: 1-3 X SSPE/SSC

- A blocking agent such as Denhardt's reagent or BLOTTO (non-fat, dried milk) can be added in either the prehybridization and/or hybridization step.
- Optionally, 50% formamide can also be added, although this may decrease the hybridization rate compared to aqueous solutions.
  NOTE: The use of formamide may destroy the interaction between biotin and streptavidin. See section G.2.1.

Washing conditions The washing conditions should be as stringent as possible to minimize background problems.
A combination of temperature and salt concentration should be chosen that is approx. 5-10°C below the calculated T_m of the hybrid under study (3).
At least four 15 minutes washes are recommended.

Elution conditions Heating, alkali denaturation or change in ionic strength is recommended. Be aware that heating may be destroy the interaction between biotin and streptavidin.

Hybridization time Hybridization time of DNA/RNA probes to targets will vary greatly depending on the type of hybridization reaction being performed.

Equations for T_m DNA:DNA hybrids (Bolton and McCarthy 1962)
$T_m = 81.5°C + 16.6(\log_{10}[Na^+]) + 0.41\ (\%G+C) - 0.63(\%\ formamide) - (600/N)$

DNA:RNA hybrids (Casey and Davidson 1977)
$Tm = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58\ (\%G+C) + 11.8\ (\%G+C)^2 - 0.50(\%\ formamide) - (820/N)$

RNA:RNA hybrids (Bodkin and Knudson 1985)
$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58\ (\%G+C) + 11.8\ (\%G+C)^2 - 0.35(\%\ formamide) - (820/N)$

where N = length of hybrid in base pairs

The relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable).

Mismatched base Hybridization rate is reduced by a factor of two for each 10% of mismatched base pairs.

Duplex length As the duplex length increases, the hybridization rate increases proportionally.

Viscosity Increased viscosity reduces the hybridization rate when using Dynabeads.

5.9 Technical tips

DNA fragments more than 40 base pairs or more complex solutions (e.g. high level of impurities/non-specific DNA) need a longer hybridization time.

The tube/tray must be placed on a rotation device to avoid sedimentation of the beads (hybridization oven).

Make sure all buffers and equipment are free of RNAses when working with RNA.

5.10 Buffers and solutions

RT-buffer:
50 mM Tris-HCl pH 8.3
100 mM KCl
10 mM $MgCl_2$
5 mM DTT
2 mM dNTP (500 mM each);

Binding and Washing buffer
1M NaCl
10 mM Tris-HCl pH 7.5
1 mM EDTA

50 X Denhardt's mix
10% (w/v) Ficoll
10% (w/v) PVP polyvinylpyrolidone
10% (w/v) BSA

6X SSPE
500 mM NaCl
33 mM $NaH_2PO_4xH_2O$
3.3 mM EDTA

Dissolve the reagents in 800 ml of H_2O. Adjust the pH to 7.4 with NaOH. Adjust the volume to 1 litre with H_2O. Sterilise by autoclaving.

6X SSC
0.9 M NaCl
0.09 M sodium citrate

Dissolve the reagents in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 10 M solution of NaOH. Adjust the volume to 1 litre with H_2O. Sterilize by autoclaving.

Hybridization mix
2x SSC or 6x SSC
0.1% SDS
1x Denhardt's mix

Hybrid wash
0.1 x SSC
0.1% SDS

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6. DIRECT PURIFICATION OF M13 SINGLE-STRANDED DNA TEMPLATES FOR DNA SEQUENCING

6.1 General introduction

This protocol is designed for direct purification of M13 single-stranded DNA templates from cleared phage supernatant, to yield template for one to four sequencing reactions.
The general template purification scheme involves M13 phage lysis and hybridization of the M13 DNA to Dynabeads lacZ. Dynabeads lacZ are Dynabeads M-280 Streptavidin coated with an oligonucleotide primer complementary to the (+) strand of the M13 vector. This oligonucleotide is covalently bound to a biotin molecule and joined to the Dynabeads via the high-affinity biotin-streptavidin linkage. It is complementary to the lacZ region present in vectors such as M13 and M13-derived vectors, phagemids and double-stranded vectors. Selectivity of the purification is achieved through specific hybridization of the (+) strand M13 DNA to the Dynabeads lacZ and stringent washing conditions. The purified M13 DNA is eluted off the magnetic beads by heat denaturation, free of contaminants, and ready for DNA sequencing (1). The yield of purified template DNA should be at least 1 µg per mg Dynabeads lacZ. Use of the Dynabeads lacZ provides a significant time saving by eliminating the need for lengthy centrifugation, filtration, organic reagents, and ethanol precipitations.
The high purity of M13 gives lower background on your autoradiograph/fluorogram which results in longer read lengths.
The outlined protocol has also been automated on the CATALYST™ 800 Molecular Biology-LabStation supplied by Perkin Elmer - Applied Biosystems Division (2, 3).

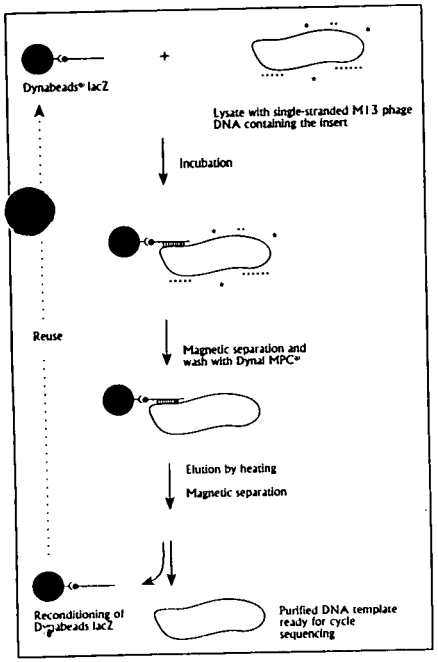

Figure 6.1 A schematic drawing for magnetic isolation of *lacZ* containing vectors.

Note: The immobilized oligonucelotide is complementary to the (+) strand of M13 vectors. Therefore, use only M13-derived vectors that package the (+) strand in the phage particles, and sequence using the forward primer.

Note: If the amount and purity of the isolated template are checked on an agarose gel, a small amount of a low molecular band may be observed. This is excess *lacZ*-oligo. This will *not* disturb subsequent sequencing reactions, as the oligo is 3'-aminylated to block any potential 3' exonuclease digestion and extension.

6.2 Materials required

- Dynabeads lacZ (2 x 1.3 ml (supplied)* or 10 ml (10 mg/ml) supplied in storage buffer)
- Magnetic Particle Concentrator - Dynal MPC (see Appendix B)
- Minimal medium
- YT medium
- Precipitation mix
- Lysis buffer (supplied)*
- Hybridization solution (supplied)*
- 3x Wash buffer (supplied)*
- Elution buffer (supplied)*
- 3x Reconditioning solution (supplied)*
- Storage buffer (supplied)*
- Control template (supplied)*
- Phage host cell (i.e. F(+) strains)
- Table top centrifuge for 1.5 ml tubes
- Heat block (42°C and 70°C)
- Sterile 1.5 ml microcentrifuge tubes
- Sterile distilled water

*) Supplied with the Dynabeads lacZ ssM13 Purification Kit (Prod.No. 625.01)

6.3 Protocols

100 µl (1 mg) Dynabeads lacZ is necessary for the purification of M13 single-stranded DNA from cleared phage supernatant to yield template for one sequencing reaction (see protocol 6.3.1).
To purify template from low-yield M13 systems, 50 µl (0.5 mg) Dynabeads lacZ is sufficient per sequencing reaction (see protocol 6.3.2). This protocol is very similar to protocol 6.3.1. The major differences are that a precipitation step is required to isolate more template, and sample and material volumes have been adjusted.
Reconditioning of Dynabeads lacZ for reuse is described in protocol 6.3.4.

6.3.1 Purification of M13 single-stranded DNA from cleared phage supernatant yielding template for one sequencing reaction.

Phage culture method
In general, phage cultures are grown in YT medium according to standard protocols. It should be noted that media containing glycerol (e.g. Terrific Broth) are not recommended for use with this protocol.

Note: A modified protocol designed for low-yield M13 systems is described in protocol 6.3.2.

1. Grow a culture of a freshly plated M13 host (i.e. bacteria producing the F pilus) in minimal media until late log phase of the growth cycle (usually $OD_{600} = 1.0$). To obtain the best results with this protocol, use both high-expression phage particle vectors and bacterial host strains.
   Note: JM109 and MV1190 are the recommended host strains.
2. Select a clear, colourless M13 plaque. Using a sterile tooth pick, inoculate 1.5 ml of YT medium containing 7 µl of the fresh plating culture.
3. Grow cultures at 37°C for a minimum of 5 hours, and not longer than 18 hours.
4. Transfer each phage culture to a separate 1.5 ml microcentrifuge tube and pellet the [illegible]eria (10,000 g for 5 minutes).
5. Transfer 600 µl of the cleared supernatant to a sterile 1.5 ml tube. Cultures may be stored on ice, or kept at 4°C, until processed further. Dynal recommends that cleared supernatants should be used within one week.

Control reaction
1. Transfer 500 µl of YT media to a new 1.5 ml tube.
2. Add 10 µl (2 µg) control template (e.g. M13mp18) to the media.
3. Follow the rest of the protocol as stated.

Lysis
1. Add 80 µl of lysis buffer to the tube containing the cleared supernatant (from step 5 of the phage culture method; from step 2 under control reaction).
2. Mix by gently inverting the tube several times and incubate at 70°C for 15 minutes.
   Note: The lysis buffer will form a precipate when stored at 4°C. This precipitate is easily dissolved by warming the buffer prior to use.

Hybridization
1. Add 350 µl of hybridization solution to the lysed culture.
2. Add 100 µl of Dynabeads lacZ (from the original stock or from step 8 of the reconditioning Dynabeads lacZ protocol in section 6.3.4) to each sample containing the lysed culture and [illegible]ridization solution.
   Note: Remember to resuspend the Dynabeads lacZ by gently shaking the tube to obtain a homogeneous suspension prior to transfer.
3. Mix by inversion and incubate at 42°C for 30 minutes. Mix once again halfway through this incubation.

Wash
1. If necessary, spin briefly (7 seconds) to bring down condensation on the sides of the tube. Place the tube in the Dynal MPC.
2. When the Dynabeads lacZ have been pulled to the side of the tube (after approximately 1 minute), aspirate and discard the liquid. Avoid touching the inside wall of the tube where the Dynabeads are attracted to the magnet.
3. Dilute 3x wash buffer with distilled water to 1x concentration. Prepare enough 1x wash buffer for all the samples (600 µl per sample plus an additional 200 µl needed for the recycling of the Dynabeads lacZ).
4. Remove the tubes from the Dynal MPC and add 300 µl of the prepared 1x wash buffer.
5. Mix by inversion, and place the tubes in the Dynal MPC. When the beads have been pulled to the side of the tube (after approximately 30 seconds), remove and discard the supernatant.

6. Repeat step 4 and 5 so that the beads are washed twice.
7. Remove the supernatant from the beads and discard.

Elution
1. Add 10-30 µl (use 20 µl initially) of elution buffer to the Dynabeads lacZ and mix by pipetting. Incubate at 70°C for 5 minutes to separate the hybridized strands.
2. If necessary, spin briefly. Place the tube back into the Dynal MPC.
   Note: Step 1 and 2 must be performed as quickly as possible to prevent the reannealing of the two strands.
3. Transfer the liquid containing the eluted DNA template to a sterile tube. The eluted DNA template is now ready for sequencing.
4. Recondition the Dynabeads lacZ as described in the reconditioning protocol (see section 6.3.4) and store in storage buffer at 4°C using a separate tube.
   Note: The Dynabeads lacZ must be maintained in liquid during storage and all handling steps. Drying of the Dynabeads will result in reduced performance.

Sequencing reactions
Adjust the volume of purified template with water according to the template volume of your sequencing protocol. Continue the sequencing protocol as described by the manufacturer.

Volumes of template required for extension reactions will vary depending on which chemistry is used.

If using any of the PRISM™ AmpliTaq® Cycle Sequencing Kits supplied by the Applied Biosystems Division of the Perkin-Elmer Corporation, the following volumes are recommended:

- For *Taq* dye terminator sequencing, use 5.0 µl of template per reaction.
- For *Taq* dye primer sequencing, use 1.0 µl of template for A and C reactions, and 2.0 µl of template for G and T reactions.

If the yield of DNA template is sufficiently high, T7 DNA polymerase (Sequenase®) may also be used as the sequencing enzyme.

6.3.2 Purification of M13 single-stranded DNA from precipitated phage pellet yielding template for several (up to 3) sequencing reactions.

This protocol describes how to purify template from low-yield M13 systems. Materials for this modified protocol are the same as those described for the standard protocol, except for the addition of a precipitation mix.

Note: Prepare the precipitation mix in advance. You will need a minimum of 240 µl for each 1.2 ml of phage culture supernatant. Different volumes can be scaled up or down proportionally.

Phage culture method
Follow the method described in protocol 6.3.1.

Preparation of Dynabeads lacZ
For each sample, prepare 50 µl of Dynabeads lacZ as follows.

1. Mix the stock tube of Dynabeads lacZ before dispensing to make sure the beads are uniformly distributed.
2. Pipet 50 µl of Dynabeads lacZ (per sample) into a sterile 1.5 ml tube and place in the Dynal MPC.
3. After the beads are pulled to the side of the tube (after approximately 30 seconds), aspirate the liquid. Add 50 µl of hybridization buffer to the beads and mix briefly. The beads are now ready for the hybridization step.

Phage isolation

1. Transfer each phage culture (1.4 ml) to a separate 1.5 ml microcentrifuge tube and pellet the bacteria (10,000 g for 5 minutes).
2. Transfer 1.2 ml of the cleared phage supernatant to a new 1.5 ml tube and add 240 µl of precipitation mix.
3. Mix the tube by inversion and place on ice for 30 minutes.
4. Pellet the precipitated phage particles in a microcentrifuge (10,000 g for 7 minutes). Aspirate and discard the liquid.

Control reaction

1. Add 10 µl (2 µg) control template (e.g. M13mp18) to a 1.5 ml tube.
2. Follow the rest of the protocol as stated.

Lysis

1. Resuspend the phage pellet in 25 µl of distilled water and then add 50 µl of lysis buffer.
2. Mix by inverting the tube several times and then incubate at 70°C for 15 minutes.
   Note: The lysis buffer will form a precipate when stored at 4°C. This precipitate is easily dissolved by warming the buffer prior to use.

Hybridization

1. Add 50 µl of prepared Dynabeads lacZ to each sample containing the lysed culture and hybridization solution.
   Note: Remember to resuspend the Dynabeads lacZ by gently shaking the tube to obtain a homogeneous suspension prior to transfer.
3. Mix by inversion and incubate at 42°C for 30 minutes. Mix once again halfway through this incubation.

Wash

1. If necessary, spin briefly (7 seconds) to collect the condensation from the sides of the tube. Place the tube in the Dynal MPC.
2. After the beads have been pulled to the side of the tube (after approximately 30 seconds), aspirate and discard the liquid.
3. Remove the tubes from the Dynal MPC and add 150 µl of 1x wash buffer along the side of the tube.
4. Mix the tube by inversion, and place the tubes in the Dynal MPC. When the beads have been pulled to the side of the tube (after approximately 30 seconds), remove and discard the supernatant.
5. Repeat step 1 through 4 so that the beads are washed twice.
6. Remove the supernatant from the beads and discard.

Elution

1. Pipet 10-30 µl (use 20 µl initially) of elution buffer into each tube.
2. Mix by pipetting and then incubate at 70°C for 5 minutes. If necessary, spin briefly. Place the tubes back into the Dynal MPC.
   Note: Step 1 and 2 must be performed as quickly as possible to prevent the reannealing of the two strands.
3. Transfer the liquid containing the eluted DNA template to a sterile tube. The eluted DNA template is now ready for sequencing.
4. Recondition the Dynabeads lacZ as described in the reconditioning protocol (see section 6.3.4) and store in storage buffer at 4°C using a separate tube.

Note: The Dynabeads lacZ must be maintained in liquid during storage and all handling steps. Drying of the Dynabeads will result in reduced performance.

Sequencing reactions

Follow the recommendations described in protocol 6.3.1.

6.3.3 Purification of single-stranded DNA from double-stranded plasmid DNA yielding template for one sequencing reaction.

Minipreparations of plasmid DNA (from 1.5 ml culture) obtained either by the alkaline lysis method or by the boiling method (4) can be used as starting material for this protocol. The double-stranded DNA must be denatured by heating prior to hybridization.

1. Boil the double-stranded DNA for 5 minutes.
2. Immediately, place the tube on ice.
3. Continue the preparation using protocol 6.3.1. Start protocol 6.3.1 at the hybridization step (step 1).

6.3.4 Reconditioning of Dynabeads lacZ

After template elution, the Dynabeads lacZ can be reconditioned and reliably used for four additional template purification cycles. Record each use of the beads and discard the beads after the fifth round of purification. The Dynabeads lacZ must be kept hydrated to maintain activity. At any point in the protocol, the beads can be stored temporarily in 1x wash buffer.

Dilute the 3x reconditioning solution with distilled water to 1x concentration. Prepare enough 1x reconditioning solution for all your samples (200 µl per samples).

Procedure

1. Transfer 100 µl of 1x reconditioning solution to each tube containing used Dynabeads lacZ, and resuspend.
2. Pool the resuspended beads into a new 1.5 ml tube.
3. Vortex the tube briefly and incubate at room temperature for 1 minute.
4. Spin the tube briefly in a microcentrifuge. Collect the Dynabeads lacZ at the side of the tube with the Dynal MPC and decant the liquid.
5. Resuspend the Dynabeads lacZ in 1x reconditioning solution. Use half of the volume of 1x reconditioning solution that was used in step 1 (i.e., 50 µl times the number of tubes).
6. Repeat step 3 and 4.
7. Resuspend the Dynabeads lacZ in an equal amount of 1x wash buffer (i.e., 100 µl times the number of tubes), decant, and repeat once more.
8. Finally, resuspend the recycled Dynabeads lacZ to the full original volume in the storage buffer, The final volume of resuspension should be equivalent to 100 µl per sample used. Store the recycled Dynabeads lacZ at 4°C.

After this treatment, the Dynabeads lacZ are reconditioned and ready for another purification cycle. The Dynabeads lacZ are good for at least five purification cycles (the first, plus four reconditionings).

6.4 M13mp18 information

The sequence of the lacZ oligo (40-mer) is:

5' TT ATC CGC TCA CAA TTC CAC ACA ACA TAC GAG CCG GAA GC 3'

The oligo is 3'-aminylated to block any potential 3' exonuclease digestion and extension.

According to the complete nucleotide sequence of M13mp18 first described by Yanisch-Perron *et al.* (5), the lacZ oligo is located complementary to the bases 6193 to 6154.

Sequence

The sequence of the recommended forward (-21) sequencing primer and the sequence of M13mp18 for the first 640 bases from the priming site are shown below. The complete sequence of M13mp18 can be found in Yanisch-Perron *et al.* (5).

Forward (-21) sequencing primer:

5' TGT AAA ACG ACG GCC AGT 3'

Partial M13mp18 sequence (first 640 bases from the priming site):

```
5'...
GCCAAGCTTG  CATGCCTGCA  GGTCGACTCT  AGAGGATCCC    40
CGGGTACCGA  GCTCGAATTC  GTAATCATGG  TCATAGCTGT    80
TTCCTGTGTG  AAATTGTTAT  CCGCTCACAA  TTCCACACAA   120
CATACGAGCC  GGAAGCATAA  AGTGTAAAGC  CTGGGGTGCC   160
TAATGAGTGA  GCTAACTCAC  ATTAATTGCG  TTGCGCTCAC   200
TGCCCGCTTT  CCAGTCGGGA  AACCTGTCGT  GCCAGCTGCA   240
TTAATGAATC  GGCCAACGCG  CGGGGAGAGG  CGGTTTGCGT   280
ATTGGGCGCC  AGGGTGGTTT  TTCTTTTCAC  CAGTGAGACG   320
GGCAACAGCT  GATTGCCCTT  CACCGCCTGG  CCCTGAGAGA   360
GTTGCAGCAA  GCGGTCCACG  CTGGTTTGCC  CCAGCAGGCG   400
AAAATCCTGT  TTGATGGTGG  TTCCGAAATC  GGCAAAATCC   440
CTTATAAATC  AAAAGAATAG  CCCGAGATAG  GGTTGAGTGT   480
TGTTCCAGTT  TGGAACAAGA  GTCCACTATT  AAAGAACGTG   520
GACTCCAACG  TCAAAGGGCG  AAAAACCGTC  TATCAGGGCG   560
ATGGCCCACT  ACGTGAACCA  TCACCCAAAT  CAAGTTTTTT   600
GTCGAGG     TGCCGTAAAG  CACTAAATCG  GAACCCTAAA   640
                                    ...3'
```


6.5 Buffers and solutions

Minimal medium	
YT medium	To 900 ml of deionized H_2O, add: 8.0 g bacto-tryptone 5.0 g bacto-yeast extract 2.5 g NaCl Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5M NaOH. Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving.
Precipitation mix	3.0 M NaCl 25% PEG_{6000}
Lysis buffer (supplied)*	125 mM EDTA, pH 8.0 1.5% SDS
Hybridization solution (supplied)*	2.5 M $NaClO_4$ 12% PEG_{6000}
3x Wash buffer (supplied)*	120 mM Tris-HCl, pH 8.0 0.2 mM EDTA 0.05% Triton X-100
Elution buffer (supplied)*	10 mM Tris-HCl, pH 9.0 0.1 mM EDTA 0.02% Triton X-100
3x Reconditioning solution (supplied)*	0.5 M NaOH 0.06% Triton X-100
Storage buffer (supplied)*	250 mM Tris-HCl, pH 8.0 20 mM EDTA 0.1% BSA 0.02% sodium azide
Control template (supplied)*	(25 µl M13mp18, 0.2 µg/µl)

*) Supplied with the Dynabeads lacZ ssM13 Purification Kit (Prod.No. 625.01)

6.6 References

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7. SAMPLE PREPARATION
Magnetic isolation of cells and bacteria for DNA and RNA work

7.1 General introduction

In vitro amplification of DNA derived from foodborne pathogens or pathogens from clinical specimens, such as blood, urine and feces, requires initial sample preparation to remove polymerase inhibitors and to prepare a sample volume suitable for PCR. Traditional sample preparation often includes steps such as phenol extraction, alcohol precipitations and several centrifugation steps which are not simple to standardize and automate.

Dynabeads products can quickly prepare high quality samples for downstream molecular biology applications. Dynal has created specific products for isolation of cells and bacteria.

These products are based on the same biomagnetic separation technology as the Dynal molecular biology products described in this handbook. Dynabeads are coated with antibodies against a specific cell type or bacteria. The cell or bacteria bound to the antibody coated Dynabeads are isolated and washed using a magnet (Dynal MPC).

The Dynabeads system is a gentle capture method for microbial pathogens. The technique enables both purification and concentration of target cells. Together with a PCR assay (colourimetric- or electrophoresis-based detection step), the Dynabeads system provides a sensitive method, suitable for large-scale and routine clinical diagnostic work (1, 2).

After isolation of the target organisms, microwave heating or boiling is generally sufficient for preparing template DNA. Freeze-thawing and snap-cooling of the samples can also be employed to open cells and make the DNA available for PCR amplification. Samples that have been frozen often contain nonviable cells, but these cells can still be extracted with the immunomagnetic technique.

Once the cells or bacteria are isolated with the Dynabeads products, they can be used directly in the downstream molecular biology application. Cell and bacteria can be isolated in approximately 30 minutes.

Using the Dynabeads system, it is possible to use one Dynabeads product to isolate pure cells, and then use a second Dynabeads product in the desired molecular biology application. For example, mRNA can be successfully isolated from monocytes using Dynabeads M-450 CD14 and Dynabeads Oligo $(dT)_{25}$. The pure monocytes are isolated from whole blood using Dynabeads M-450 CD14. The isolated cells are lysed and the Dynabeads are removed from the supernatant. Dynabeads Oligo $(dT)_{25}$ are then added to the supernatant to isolate pure mRNA. (For further details on mRNA isolation see chapter 2.)

Dynabeads products can be used as a pre-PCR step to isolate bacteria from various samples. For example, *Shigella dysenteriae* type 1 and *S. flexneri* can be isolated directly from feces using Dynabeads products (2). After capture, the bead-bound bacteria are boiled or freeze thawed to release the DNA for PCR. PCR amplified DNA can be detected by gel electrophoresis and dot blot hybridization.

Because of the flexibility of the Dynabeads system, in many instances, several Dynabeads products will suit the needs of a particular application.

Several products can be used for isolation of more than one type of cells. The first group of products are **Dynabeads coated with antibodies specific** for human leukocytes or specific bacteria (i.e. *Salmonella, E. coli*). This group of primary coated Dynabeads products are specific in their applications. These products are cost efficient and convenient because of the high quality antibodies precoated on the surface of the Dynabeads.

The second group are **Dynabeads coated with general polyclonal antibodies** that recognize either rat, mouse or rabbit primary antibodies. This group of secondary coated Dynabeads

products are extremely flexible and can be used in many research applications. Specific rat, mouse or rabbit primary immunoglobulins can be easily bound to one of these products. The cell/bacteria type isolated by these products are determined by the specificity of the primary immunoglobulin.

The third type of product is **Dynabeads M-280 Streptavidin.** Any biotinylated ligand can be coupled to streptavidin coated Dynabeads and subsequently be used for cell/bacteria isolation. Once again the cell type isolated by this product is determined by the specificity of the biotinylated ligand.

The last group of products are **uncoated and pre-activated Dynabeads products.** If none of the precoated Dynabeads product meet your needs, a specific ligand can be conveniently coupled to the Dynabead surface. The ligands are either physically adsorbed on the surface of Dynabeads M-450 Uncoated or chemically coupled to the surface of the tosylactivated Dynabeads products. Cells isolated by the Dynabeads technique are of high purity and viability (e.g., >98% purity for isolated reticulocytes or megakaryocytes (3, 4)).

For more detailed description of these Dynabeads products, please contact your local Dynal office or Dynal distributor.

7.2 Cells isolated using Dynabeads products

Hundreds of cell types have been isolated using Dynabeads products, including common human leukocytes (5, 6) (e.g., CD2, CD4, CD8 and CD19 positive cells), stem cells (7), endothelial cells (8), osteoclasts (9) and eosinophils (10). In addition, pure lymphocytes have been isolated from mice using Dynabeads M-280 Streptavidin (11) and CD34 positive cells have been isolated from Rhesus monkey using Dynabeads M-450 CD34.

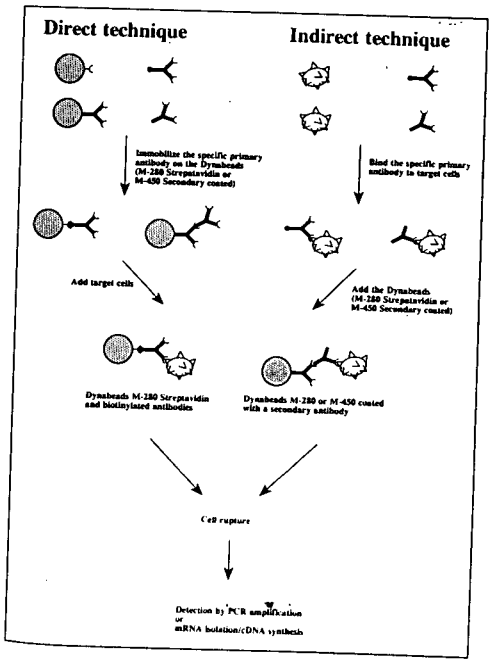

Figure 7.1 The principle of direct and indirect isolation of cells, using Dynabeads M-280 Streptavidin or a secondary coated Dynabeads M-450.

Cell isolation using Dynabeads products is a simple and easy procedure and can be performed using either a direct or an indirect technique (see Figure 7.1). For detailed protocols, please contact your local Dynal office or Dynal distributor.

7.3 Microbial pathogens isolated using Dynabeads products

Dynabeads products have been used successfully in the detection of pathogens in urine (12), food and water samples (13), blood (14) and feces (2).

Dynabeads products have been used to enrich for *Salmonella* (15), *Escherichia coli* O157 (16), *Chlamydia trachomatis* (12), the malaria causing parasite *Plasmodium falciparum* (14) and many more (2).

Once isolated, the pathogen can be used directly in a PCR-based detection system.

Isolation of specific bacteria using the primary coated Dynabeads products is simple and easy. A brief description of the methodology is shown in Figure 7.2. Once isolated, the bacteria can be used directly in a desired molecular biology application. For a detailed description, please contact Dynal.

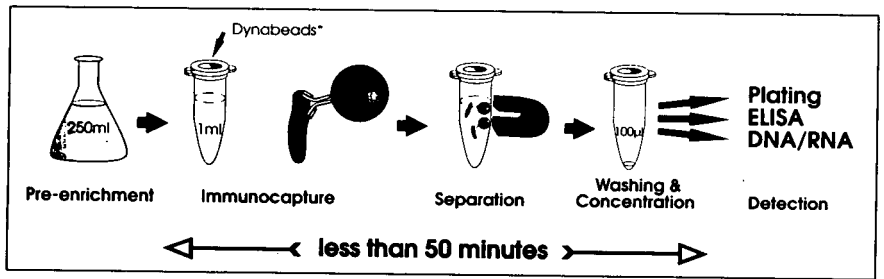

Figure 7.2 The principle of bacterial isolation using primary coated Dynabeads.

7.4 DNA/RNA capture using Dynabeads products

An alternative to antibody-coated Dynabeads for sample preparation is to use a nucleic acid capture probe coupled to the Dynabead surface (see Figure 7.3). Solid-phase captured single-stranded nucleic acids can be purified and concentrated by magnetic separation (described in more detail in chapter 5).
Biotinylated specific nucleic acid probes in combination with Dynabeads M-280 Streptavidin have been used for the recovery of viral RNA (17) and the isolation of individual tRNA (18). Dynabeads Oligo $(dT)_{25}$ have been used for detection of polyadenylated HIV-1 RNA genomes (19).

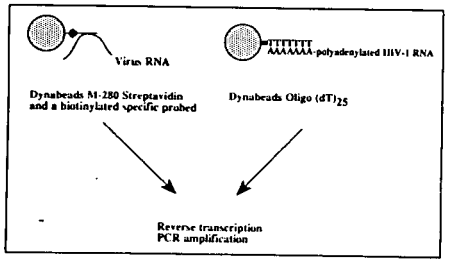

Figure 7.3 Isolation of nucleic acids using Dynabeads products with a coupled nucleic acid probe.

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8. IMMUNOMAGNETIC CELL SEPARATION USING DYNABEADS M-280 STREPTAVIDIN

8.1 General introduction

Biotinylated antibodies bound to Dynabeads M-280 Streptavidin provide a simple and rapid method for the positive isolation or negative depletion of cells. Cells can for example be isolated from whole blood, buffy coat or gradient isolated mononuclear cells.

The widespread availability of commercially produced biotinylated antibodies makes Dynabeads M-280 Streptavidin particularly suitable for immunomagnetic cell separation. When performing cell depletions, a cocktail of biotinylated antibodies can be used with Dynabeads M-280 Streptavidin.

Extremely fragile cell types may benefit from a slower attraction speed of Dynabeads M-280 towards the Dynal MPC.

For cell separation using non-biotinylated antibodies, Dynal offers Dynabeads coated with various secondary antibodies in both the M-450 (4.5 μm) and M-280 (2.8 μm) sizes (see chapter 7). Tosylactivated Dynabeads are also available for coating with your own secondary antibody.

8.2 Materials required

- Dynabeads M-280 Streptavidin
- Magnetic Particle Concentrator - Dynal MPC (see Appendix B)
- Biotinylated antibodies/Antibodies
- Biotin-X-NHS-Ester (MW=454.5)
- DMSO
- 1.0 M $NaHCO_3$
- Biogel P-30
- PBS
- 0.1 M NaN_3
- 0.1% BSA

Note: Buffers and solutions are described in section 8.6

8.3 Protocols

8.3.1 Preparation of biotinylated antibodies

The large selection of commercially available biotinylated antibodies eliminates in many cases the need to biotinylate antibodies. If the antibody you wish to use is not available in a biotinylated form, you can biotinylate it yourself using the following protocol (1, 2).

Note: The antibody you wish to use should either be a purified monoclonal or an affinity-purified polyclonal antibody. Sera are unsuitable for biotinylation.

1. Calculate the number of purified antibody molecules per volume unit.
2. Dissolve 10x molar excess of the biotinylated reagent Biotin-X-NHS-Ester (MW=454.5) in 10 μl DMSO and add this solution to the antibody solution. (For more information about recommended biotin, see appendix E.)

3. Add the required amount of a 1.0 M $NaHCO_3$ stock solution, pH 8.0 to obtain a final concentration of 0.1 M. Check pH and adjust to 8.0 if necessary.
4. Incubate overnight at 4°C.
5. Filter on a gel, e.g. Biogel P-30 in PBS with 0.1 M NaN_3 (final concentration).
6. Calculate the final concentration of antibodies and store at 4°C. BSA or a similar protein should be added to a final concentration of 0.1% to stabilize the antibody during storage.

8.3.2 Cell separation

Direct vs. Indirect method

Using the Dynabeads immunomagnetic system, a target cell or molecule can be isolated with a direct or indirect technique. With the ***direct technique***, a biotinylated antibody, specific for the target cell, is coupled to the Dynabeads M-280 Streptavidin. This complex is then used to capture the desired target.

8.3.2.1 Direct binding of biotinylated antibodies to Dynabeads M-280 Streptavidin - Direct technique

1. Before use, wash the Dynabeads as specified in Appendix D or in the product insert.
2. Calculate the amount of biotinylated antibodies needed. Between 5-10 μg antibodies per mg Dynabeads is sufficient when saturation of the streptavidin is desired and assuming 100% biotinylation of the antibody.
3. Incubate at room temperature for 30 minutes with gentle rotation of the tube (e.g. Dynal sample mixer).
4. Separate the Dynabeads M-280 Streptavidin, now coated with biotinylated antibodies, using a Dynal MPC for 1-2 min.
5. Wash 4-5 times in PBS/BSA using a Dynal MPC.
6. Resuspend to the desired concentration.

Using the ***indirect technique***, the biotinylated antibody is added first to the heterogenous suspension containing the desired target cell. Following binding of the antibody to the target cell and washing of the cells to remove excess of antibody, the Dynabeads M-280 Streptavidin are added to capture the antibody/cell complex. The captured complex is separated from the heterogeneous suspension using the Dynal MPC.

8.4 Reference applications

Dynabeads M-280 Streptavidin has been successfully used in the positive isolation of fetal mice T cell receptor Vγ3 thymocytes (3, 4), mice CD4+Vβ8+ T cells from spleen cells (5), mice CD4+ T cells from lymph nodes (6), fetal mice T cell receptor Vγ3 skin cells (7) and mice $\gamma\delta$ T cells from lungs (8).

The use of Dynabeads M-280 Streptavidin for depletion of CD4+ and CD8+ cells from spleen and lymph node cells has been reported by Qin *et al.* (9).

The same principle can be used at the molecular level to isolate and purify specific receptors as described by Ahmed *et al.* (10). Using a functionally unimpaired, cleavable, biotinylated derivative of anti-melanocyte-stimulating hormone (α-MSH), the α-MSH receptor could be recovered after photolysis, homogenization and membrane solubilization of the isolated cultured melanoma cells. After magnetic capture of the Dynabeads M-280 Streptavidin-biotin-α-MSH-receptor complex, the α-MSH-receptor complex could be detached by cleavage.

Wittig *et al.* (11) have used a biotinylated monoclonal antibody against the zigzag backbone of Z-DNA for identification of Z-DNA in individual genes. The biotin labeled Z-DNA specific monoclonal antibody was allowed to diffuse inside the nucleus, which was permeabilized by detergent treatment of agarose encapsuled cells. The antibody was crosslinked to Z-DNA with UV-laser, the DNA restricted with Alu I and the protected Z-DNA isolated using Dynabeads M-280 Streptavidin. The DNA bound antibody was recovered by digestion with Proteinase K and the released DNA was PCR amplified and electrophoretically separated on polyacrylamide gel, blotted and hybridized with radioactive probes.

8.5 Technical Tips

In all immunological techniques, the effectiveness of immunomagnetic separation using Dynabeads is dependent on several parameters:

- ***Bead concentration***
  A minimum of 10^7 beads per ml of solution during the incubation period should be used in both the direct and indirect method. This is the most critical parameter to achieve a successful isolation.

- ***Number of Dynabeads per target cell***
  For a positive selection, a minimum bead to target cell ratio of 4:1 is recommended. The ratio can be optimized down to as little as 1:1.

- ***Cell concentration***
  Whenever possible, a total cell concentration of $1x10^7$–$4x10^7$ cells/ml should be used.

- ***Incubation time and temperature***
  During the incubation of Dynabeads with the target cells, Dynal recommends an incubation time between 5–60 minutes at 4°C.

- ***Bead agitation***
  During the incubation step, the Dynabeads and cells should be continuously mixed using bidirectional rotation (i.e. using the Dynal Sample Mixer).

- ***Sample volume***
  The actual volume of the sample is not crucial, but the sample volume in relation to the tube size should be approximately 1/2 to 3/4 of the tube volume.

- ***Detachment***
  Various methods may be employed to detach the isolated target cells from the beads. Anti-F_{ab} antibodies, DETACHaBEAD manufactured by Dynal have been used to overcome the binding between antibody and antigen. In some cases, an overnight incubation at 37°C in a standard culture medium may lead to an adequate degree of detachment. Addition of 2% EDTA into a standard elution medium may also cause detachment within 30 minutes. Enzymatic treatments have also been employed for detachment of cells from Dynabeads.

- ***Magnetic separation***
  Compared to Dynabeads M-450 (4.5 μm), Dynabeads M-280 (2.8 μm) are smaller in size and have a lower magnetic susceptibility. Therefore, to ensure a proper separation of Dynabeads from solution, Dynabeads M-280 may require a longer separation time on the Dynal MPC (e.g. 1-2 minutes).

8.6 Buffers and solutions

DMSO (Dimethyl sulfoxide)	
1.0 M $NaHCO_3$	
Phosphate-buffered saline (PBS buffer), pH 7.5	137 mM NaCl 2.7 mM KCl 4.3 mM $Na_2HPO_4 \cdot 7H_2O$ 1.4 mM KH_2PO_4
0.1 M NaN_3	
0.1% BSA	

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9. APPLICATIONS OF DYNABEADS M-280 STREPTAVIDIN FOR DNA/RNA BINDING PROTEINS

9.1 Purification of sequence-specific DNA/RNA binding proteins

9.1.1 General introduction

DNA/RNA binding proteins (e.g. promoters, gene regulatory proteins and transcription factors) represent a major challenge to biology both in terms of normal cellular control and in disease states such as cancer. Unfortunately, these molecules are often short-lived and in low abundance.

To study these molecules successfully, they need to be highly purified using a rapid and sensitive method. A magnetic DNA affinity purification method developed by Gabrielsen *et al.* (1, 2) describes the use of Dynabeads M-280 Streptavidin for this application. The scheme is outlined in Figure 9.1.

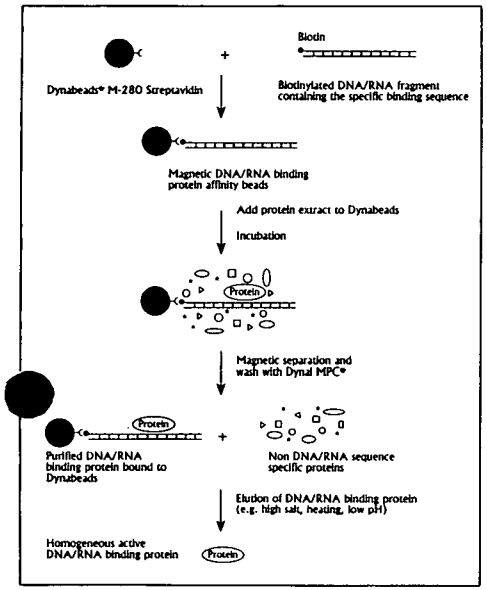

Figure 9.1 Diagram of method for purification of strand-specific DNA/RNA binding proteins using a magnetic DNA/RNA affinity bead.

In essence, one requires a biotinylated DNA/RNA sequence motif to which the protein of interest will specifically bind. When coupled to Dynabeads coated with streptavidin, it then forms a reusable, magnetic solid-phase with affinity for the target protein. The magnetic DNA/RNA affinity beads can then be mixed with the nuclear extract, and binding is completed in minutes. Subsequent washing and elution steps benefit from the speed and convenience of Dynabeads biomagnetic separation, making isolation of proteins possible in one hour. Improved purity and yields are obtained by saturating the DNA affinity beads with the specific DNA binding protein and by including competitor DNA only during washing of the beads (2), This development represents a significant improvement over classical purification techniques and has made possible studies of DNA/RNA binding proteins in highly purified form.

Dynabeads M-280 Streptavidin provide a superior alternative to affinity chromatography for the purification of low abundance, unstable, sequence specific DNA or RNA binding proteins. High yields of enriched proteins are obtained using only one adsorption step (2).

When compared to conventional purification methods (e.g. column chromatography), the Dynabeads technique has several advantages:

- **FAST** — In the case of purification of yeast transcription factor tau (τ), higher purity was obtained in less than one hour using Dynabeads than was normally obtained with the same starting material after three days and three columns (1).
- **EFFICIENT** — The high stability and binding capacity of the DNA on the Dynabeads allows binding of the target proteins with kinetics similar to that of DNA in free solution (2).
- **COST EFFECTIVE** — DNA coupled to Dynabeads M-280 Streptavidin is reusable at least ten times (3).

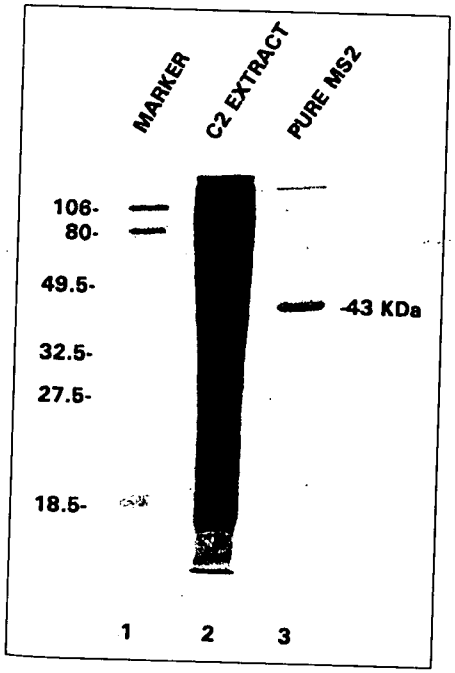

Figure 9.2 Comparison of C_2 murine skeletal myotube extract and purified MS2, a new DNA binding protein (3). Denaturing gel, 12% SDS-PAGE, stained and photographed. Lane 1: 5 μl prestained marker proteins; lane 2: 100 μg C2 cell extract; lane 3: 0.5 μg MS2 magnetically purified from C2 cell extract. MS2 is seen to be a single protein of 43 kDa.

Publications describing the use of Dynabeads M-280 Streptavidin to purify DNA/RNA binding proteins include the following results:

- Yeast transcription factor tau (τ) has been purified to near homogeneity in minutes and is fully active in transcription and DNA binding assay (1, 2).
- Purification of a new DNA binding protein, MS2, from C_2 murine skeletal myotubes has been accomplished (3).

- The ecdysteroid receptor (EcdR) from *Drosophila melanogaster* has been enriched 29,000 fold within 1.5 hour using this technique (4).

An overview of different sequence specific DNA/RNA binding proteins isolated using Dynabeads M-280 Streptavidin is mentioned in Table 9.1 below.

Table 9.1 Purification of different sequence specific DNA/RNA binding proteins using Dynabeads M-280 Streptavidin as the magnetic DNA/RNA affinity bead with belonging target binding sequences and elution conditions.

...ding protein	Target binding sequence	Elution condition	Authors	Ref.
DNA topoisomerase I	The 5'-phosphate end of a cleaved DNA strand	High conc. of dinucleotides in a high salt buffer	Alsner, J. *et al.*	5
FMR1 protein	Single-stranded **RNA**	Released by heating	Ashley Jr., C.T. *et al.*	6
Yeast transcription factor tau (τ)	Double-stranded DNA	High salt buffer	Gabrielsen, O.S. *et al.*	1
Yeast transcription factor tau (τ)	Double-stranded DNA	High salt buffer on ice	Gabrielsen, O.S. *et al.*	2
cMyb protein	Double-stranded DNA	No elution	Gabrielsen, O.S. *et al.*	7
Vaccinia virus early transcription factor (VETR)	Double-stranded DNA	High salt buffer	Gershon, P.D. *et al.*	8
LacI-β-gal fusion protein	Double-stranded DNA	DNase I treatment	Ljungquist, C. *et al.*	9
Single-stranded telomere binding protein (sTBP)	Single-stranded $TTAGGG_n$ repeats	High salt buffer	McKay, S.J. *et al.*	10
Ecdysteroid receptor (EcdR)	Double-stranded DNA	High salt buffer	Ozyhar, A. *et al.*	4
...eprotachykinin A ...PPT)	Single-stranded DNA	High salt buffer	Quinn, J.P. *et al.*	11
Hepatic protein, p27	Guanine-rich single-stranded DNA	High salt buffer	Rahat, M.A. *et al.*	12
Initiator protein RepC	Single-stranded DNA	Alkali treatment	Rasooly, A. *et al.*	13
Protein factor MS2	Double-stranded DNA	Low pH	Ren, L. *et al.*	3
Cellular transcription factor RBP-Jκ	Double-stranded DNA	High salt buffer	Zimber-Strobl, U. *et al.*	14
Cellular transcription factor RBP-Jκ	Double-stranded DNA	High salt buffer	Laux, G. *et al.*	15

For information on immunoprecipitation of proteins using Dynabeads products, please contact Dynal for separate product information.

9.1.2 Materials required

To isolate sequence-specific DNA/RNA binding proteins the following material/equipment will be required:

- Dynabeads M-280 Streptavidin (10 mg/ml)
- Magnetic Particle Concentrator - Dynal MPC (see Appendix B)
- Biotinylated DNA/RNA sequence containing the actual binding domain for your target protein (see Appendix E for biotinylation procedures)
- Binding & Washing Buffer for immobilizing the biotinylated DNA/RNA sequence to Dynabeads M-280 Streptavidin (see section 1.6 for recipe)
- Crude or partly purified protein extract
- Competitor DNA (optional)
- Protein binding buffer
- Protein wash buffer
- Protein elution buffer
- Tilting/rotation apparatus (e.g. Dynal Sample Mixer)
- Test tubes, glassware, pipettes

See Appendix F for binding capacity and optimal binding conditions for coupling of biotinylated DNA/RNA to Dynabeads M-280 Streptavidin.
When working with RNA binding proteins, it is important to prepare the Dynabeads M-280 Streptavidin for RNA work (see Appendix D.2) and to treat all solutions, plasticware and other current equipment with diethyl pyrocarbonate (DEPC) to avoid degradation of RNA by RNases (see section 2.5).
See section 9.1.3.2 for recipes of current protein binding, wash and elution buffers.

9.1.3 Protocols

9.1.3.1 Protein purification procedure

There are a number of important factors to consider when developing a protocol including the degree of partial purification required for your specific protein to be isolated. This will ultimately depend upon the following:

- the protein
- its source
- its abundance
- its binding affinity
- the amount of other contaminating DNA/RNA binding proteins
- the presence of nucleases
- the degree of partial purification
- pH and temperature dependance of binding/elution

Additionally, consideration may have to be given to salt molarities for binding, wash and elution, the effect of added competitor DNA and the saturation conditions of protein to DNA/RNA. Given the flexibility and speed of Dynabeads, optimization of reaction conditions should be readily achieved. A full and detailed review of all relevant considerations is presented by Gabrielsen *et al.* (2).
Detailed examples of binding, wash and elution conditions are given in section 9.1.3.2. The following is a general outline of the protein purification procedure.

1) Prepare the protein extract.
2) Add Dynabeads coated with the appropriate DNA/RNA sequence to the protein extract.
3) Incubate Dynabeads with the protein extract while providing bidirectional mixing (e.g. Dynal Sample Mixer).
4) Place the tube containing the protein extract and Dynabeads in a Dynal magnet (Dynal MPC).

5) Remove the supernatant and wash the Dynabeads several times with buffer using a magnet.
6) Elute the protein by resuspending Dynabeads and the bound protein in an elution buffer.
7) Separate Dynabeads and the eluted protein using a Dynal MPC.
8) Proceed with the analysis of the purified protein.

9.1.3.2 Examples of protein binding, wash and elution conditions

Gabrielsen *et al.* (2) provide some helpful hints for optimizing protein binding, washing and elution conditions. In addition, several other publications describe the following purification conditions. See Table 9.1 for the isolated DNA/RNA binding protein.

- **Gabrielsen *et al.*** (2); Yeast transcription factor tau (τ)

  Binding: Protein binding was performed in a TGED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.01% Triton® X-100) with 100 mM NaCl and mixed at 25°C for 5 minutes.

  Washing: Dynabeads were washed once with a TGED buffer containing 100 mM NaCl, once with TGED buffer containing a 10-fold excess of poly[d(I-C)] competitor DNA, and once in TGED buffer containing 100mM NaCl.

  Elution: The bound protein was eluted with 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.01% Triton X-100 and 1 M NaCl on ice.

- **Ren *et al.*** (3); Protein factor MS2

  Binding: Protein binding was performed in 5x gel shift buffer (1x gel shift buffer: 20 mM Tris-HCl, pH 7.0, 100 mM NaCl, 5 mM EDTA, 0.1% Nonidet® P-40 [NP-40], 0.5 μg/μl BSA and 5% glycerol), 1.25 μl 1 M $MgCl_2$, 0.378 ml dialysis buffer, and mixed at room temperature for 30 minutes.

  Washing: Dynabeads were washed three times with 1x gel shift buffer containing 2.5 mM $MgCl_2$ and 0.1% NP-40, but lacking BSA.

  Elution: The bound protein was eluted with 100 mM sodium acetate, pH 4.2.

- **McKay *et al.*** (10); Single-stranded telomere binding protein (sTBP)

  Binding: Protein binding was performed with 20 μg sonicated *E.coli* DNA, 15 μg oligonucleotide B575 in 1 ml 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4% glycerol, 0.1% Triton X-100, 10 mM β-mercaptoethanol, and mixed at room temperature for 20 minutes.

  Washing: Unspecified

  Elution: The bound protein was eluted with two 100 μl washes of 20 mM Tris, 1 mM EDTA, 15% glycerol, 0.05% NP-40 and 1 M NaCl.

- **Gershon *et al.*** (8); Vaccinia virus early transcription factor (VETR)

  Binding: Protein binding was performed with buffer A (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.01% NP-40, 1 mM dithiothreitol, 10% (v/v) glycerol) containing 70 mM NaCl for 30 minutes.

  Washing: Dynabeads were washed twice for 10 minutes each time with buffer A containing 100 mM KCl.

  Elution: The bound protein was incubated three times with buffer A containing 400 mM KCl and once with buffer A containing 2 M KCl.

- **Ozyhar *et al.*** (4); Ecdysteroid receptor (EcdR)

  Binding: Protein binding was performed in 20 mM Hepes/KOH, 110 mM KCl, 2 mM dithiothreitol, 1 mM EDTA, 2 mM KH_2PO_4, 10% (v/v) glycerol, pH 7.6., and mixed at 0–4°C for 30 minutes.

  Washing: Dynabeads were washed four times with 20 mM Hepes/KOH, 110 mM KCl, 2 mM dithiothreitol, 1 mM EDTA, 2 mM KH_2PO_4, 10% (v/v) glycerol, pH 7.6.

  Elution: The bound protein was eluted with 20 mM Hepes/KOH, 10% (v/v) glycerol, pH 7.6 and 400 mM KCl.

9.1.3.3 Regeneration of the DNA/RNA affinity Dynabeads for reuse

Reuse of Dynabeads M-280 Streptavidin for purification of DNA binding proteins have been described in the publications by Gabrielsen *et al.* (2) and Ren *et al.* (3).

Gabrielsen *et al.* state:

"Used beads are regenerated by repeated washes in high salt buffer (TGED with 2 M NaCl or TGED with 2 M NaCl and 6 M urea) and washed in TEN (TE buffer with 100 mM NaCl) buffer and are stored at 4°C for further use."

Ren *et al.* state:

"After elution of purified protein, the beads should be regenerated without delay, if beads are to be reused. DNA and beads were reusable at least ten times if completely washed after each use and stored at 4°C. Beads, with or without tether s4, were prepared for storage and reuse by washing twice with TE buffer, pH 8.0."

9.1.3.4 Binding capacity and storage

As an example, a 40-mer oligonucleotide primer linked to the bead surface containing one binding site for the target protein per oligo can bind approximately 2.5 μg of a 60 kDa protein per mg of Dynabeads M-280 Streptavidin.

To increase the yield, repeating specific recognition sequences can be made. Depending on the size of the binding protein, allow at least 10 randomly synthesized nucleotides between the bead surface and the start of the recognition site.

The prepared magnetic DNA affinity beads can be stored at 4°C for months without loss of DNA binding protein binding capacity. Gabrielsen *et al.* (2) have successfully used beads stored at 4°C for more than two years.

9.2 Determination of consensus DNA sequences for DNA binding proteins

The study of the regulation of gene expression requires a method for the determination of the DNA sequence that is recognized by a specific DNA binding protein. Since individual sequences of interest often represent a very small percentage of the total sequences present, a method to recover and amplify these sequences is needed. Kinzler and Vogelstein (16) describe a method where a combination of affinity selection of the DNA, immunoprecipitation and PCR amplification of the enriched DNA is performed. This selection procedure is repeated and amplified recovered sequences can be cloned and/or used as hybridization probes.

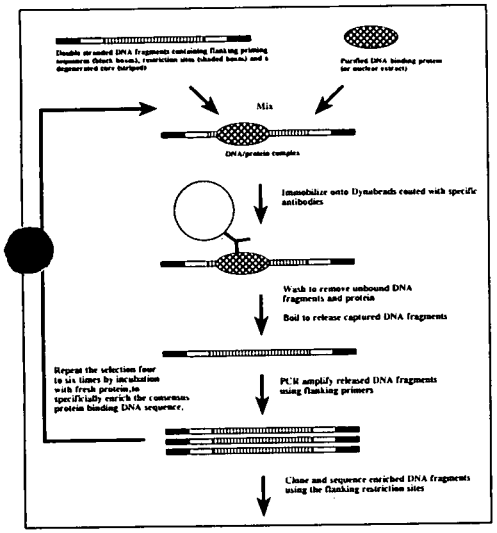

Figure 9.3 Dynabeads coated with monoclonal antibodies against DNA binding proteins can be used to investigate the consensus DNA sequence binding site, enabling fast isolation of protein-DNA complexes in the cyclic process of selective enrichment of the sequences with highest affinity.

Wright *et al.* (17, 18) describe a similar technique to determine the consensus/optimal binding site for a DNA binding protein, based on the immunocapture of the DNA/protein complex using Dynabeads coated with monoclonal antibodies specific for the DNA binding protein being studied. In this technique, termed CASTing (Cyclic Amplification and Selection of Targets), an oligonucleotide consisting of a degenerate core flanked by restriction sites and with defined sequences that can serve as priming sequences at both ends, is synthesized as a first step. The oligonucleotide is made double-stranded by priming the DNA synthesis with the 3' end primer. The resulting double-stranded DNA consists of: an upstream primer sequence-a restriction site region- the degenerated core-a second restriction site region-a downstream primer sequence. This double-stranded DNA is mixed with purified DNA binding protein or with nuclear extract and the DNA-protein complex is immunoprecipitated with Dynabeads coated with monoclonal antibodies specific for the protein of interest (anti-Ig coated Dynabeads coupled with specific monoclonal antibodies or biotinylated antibodies coupled to Dynabeads M-280 Streptavidin).

The DNA recovered from the first cycle of selection is released by boiling of the Dynabeads-protein-DNA complex and then amplified by PCR using the flanking primers. The amplified DNA is mixed with fresh protein and subjected to additional cycles of selection, including incubation with protein, immunoprecipitation and reamplification. At each cycle, the ratio of specific binding sites to random sequences is increased until sufficient specificity is obtained to justify cloning and sequencing the DNA. One cycle takes about 2.5 hours and typically four to six cycles are performed (2). The enriched DNA fragments can be easily cloned using the restriction sites introduced at each end of the degenerated core thus allowing analysis of individual sequences.

Points to consider

Each cycle enriches for the highest-affinity sites and therefore one ultimately selects for the single highest-affinity interaction. In order to observe a variety of multicomponent complexes, it is thus necessary to limit the number of cycles of selection to the minimum needed to eliminate most of the non-specific sequences.

If nuclear extracts are used as the protein source, a substantial fraction of the oligonucleotides will bind non-specifically. To reduce this problem a competitor like sonicated salmon sperm DNA (17, 18) or poly(dI-dC) (16) can be included.

Overamplification of the DNA should be avoided for two reasons. Firstly, overamplification of the DNA can result in the production of very large artifactual DNA (17) and secondly, because the population of molecules being amplified during each cycle contain common 5' and 3' end (the PCR priming sequences) but differ in the central region, reannealing of these products may produce molecules with double-stranded ends and single-stranded middles (18).

9.3 Study of multicomponent protein complexes bound to DNA/RNA

Not only can the binding properties of individual proteins and the nucleic acid sequences they recognize be studied using the Dynabeads. It is also possible to isolate and study multicomponent complexes bound to DNA in this way.

9.3.1 Complex assembly studies

Transcription initiation in eukaryotes by RNA polymerase II is preceded by the stepwise formation of a preinitiation complex (PIC). The rate of formation of PICs can govern the rate at which a gene is transcribed. Transcriptional activator proteins (activators) can increase the rate at which PICs are formed. Using Dynabeads to isolate complexes formed *in vitro* under different conditions, it has been possible to show that the yeast activator GAL4 both increases the recruitment of the general transcription factor TFIIB into PICs and accelerates a step later in assembly (19).

9.3.2 Reconstitution of chromatin on Dynabeads

In an elegant study, the group of Becker (20) has shown that it is possible to reconstitute chromatin on long (6 kb) DNA molecules attached to the Dynabeads and to use these complexes to study transcription.

9.3.3 Study of *in vivo* binding sites of DNA binding proteins

Both the detailed interaction of regulatory proteins with particular DNA sequences *in vivo* (footprinting) (21-23) as well as the *in vivo* conformation of the DNA to which regulatory proteins are bound (24), has been investigated using Dynabeads.

In the footprinting method, the Dynabeads are used to selectively capture genomic fragments generated by DNase digestion of intact nuclei. The fragments are first labelled with biotin using ligase and a biotinylated linker tag. The fragments are then amplified by PCR with Vent® polymerase and captured onto Dynabeads M-280 Streptavidin (Linker Tag Selection - LTS). See also section 12.4.2. Thereafter, the fragments are made single-stranded and used as templates for primer extension with labelled primer. The products of the extension reaction are analyzed on sequencing gels. Weak bands on the sequencing gel correspond to exposed regions of DNA devoid of protein, whereas strong bands indicate the nucleotides to which protein is intimately attached. Compared to conventional footprinting techniques, the signal strength of the footprints is greatly enhanced and background greatly reduced when Dynabeads are used to selectively enrich the genomic fragments (22).

The clarity of *in vitro* DNA footprints can also be greatly improved using Dynabeads (25, 26). To investigate DNA conformation, Dynabeads M-280 Streptavidin were used to isolate fragments of DNA to which biotinylated anti-Z-DNA antibodies had been crosslinked *in vivo*. Using this technique, it could be clearly shown that the conformational state of a myc-promoter region changed from the B- to the Z-form depending on the state of transcriptional activation of the gene (24).

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10. LABELING OF SINGLE-STRANDED PROBES

10.1 General introduction

The use of Dynabeads M-280 Streptavidin as a solid support when preparing labeled DNA probes enables purification of single-stranded templates and elution of single-stranded labeled probes without contamination of unlabeled complementary DNA. The advantage of using single-stranded probes is better hybridization results due to no competition with the complementary strand. In addition, probes generated by this method are ideal for *in situ* hybridization and other methods requiring only one labeled strand.

The probe template is synthesized and biotinylated through PCR amplification, using one biotinylated primer. The biotinylated sequence is immobilized onto Dynabeads M-280 Streptavidin and converted to a single-stranded template for production of the labeled strand. After extension and elution, the labeled single-stranded probe is ready to use for hybridization applications (1, 2).

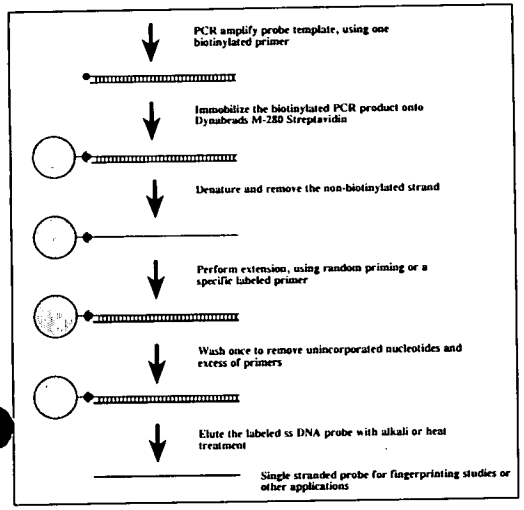

Figure 10.1 Schematic flow-chart of solid-phase synthesis of labeled single-stranded probes.

10.2 Materials required

- Dynabeads M-280 Streptavidin
- Magnetic Particle Concentrator - Dynal MPC (see Appendix B)
- Thermocycler
- PCR buffer
- Nucleotides
- *Taq* polymerase
- 6x SSC
- 1x SSC
- 10x TE buffer
- Melting solution
- Extension buffer/DNA polymerase buffer
- DNA polymerase or Standard random primer reagents
- Neutralization buffer

Note: Buffers and solutions are described in section 10.6

10.3 Protocol

1. Amplification of the probe template

This method can use any vector that can serve as PCR template.
For PCR amplification direct on whole bacteria without prior DNA purification, please refer to chapter 1 for lysis protocol.
PCR amplification can be performed on genomic DNA, using specific primers.

1.1 Add 5 pmoles of each specific primer, one biotinylated (the choice of biotinylated primer decides which strand is to be labeled), to the tube with the DNA template.
1.2 Add 50 µl of PCR buffer, containing 200 µM dNTPs, and 1 unit *Taq* DNA polymerase.
1.3 Run the PCR amplification using a PCR profile suitable for your specific primers.

2. Preparation of the Dynabeads

2.1 Use 25 µl (10 µg/µl as supplied) of washed Dynabeads M-280 Streptavidin for each probe template preparation.
2.2 Wash the Dynabeads once in 100 µl 6x SSC, and resuspend in 25 µl 6x SSC (Dynabeads may be washed in bulk, as described in Appendix C, "Preparation of Dynabeads M-280").

3. Immobilization of the PCR product

3.1 Remove the supernatant from the prewashed Dynabeads M-280 Streptavidin and add 2-10 µl of the PCR product diluted to 25 µl in 6x SSC buffer.
3.2 Incubate at room temperature for 1 minute, tap the tube a couple of times to keep the Dynabeads in suspension.
3.3 Collect the Dynabeads with the immobilized template by placing the tube in the Dynal MPC.
3.4 After at least 30 s remove the supernatant with a pipette.

Note: For longer PCR fragments (>1000bp) a longer-binding time is needed. Please see Appendix F for immobilization of nucleic acids onto Dynabeads M-280 Streptavidin and binding capacities for different lengths of DNA fragments.

4. Melting the DNA duplex

4.1 Remove the supernatant and resuspend the Dynabeads in 100 µl melting solution.
4.2 Incubate for 5 min at room temperature. The Dynabeads should be resuspended occasionally during the incubation by gently tapping the tube.
4.3 Collect the Dynabeads on the side of the tube and remove the NaOH supernatant (containing the non-biotinylated strand).
4.4 Repeat the denaturation twice with 100 µl melting buffer.
4.5 Wash the Dynabeads five times in 100 µl extension buffer (DNA polymerase buffer). Change tube after the first washing step, to make sure no NaOH droplets are left on the tube walls.

5. Labeling of the single-stranded probe

Labeling can be performed in two ways:

5a. by standard random priming reactions (commercial random primed DNA labeling Kit) (1, 3)
5b. by using a specific primer in the extension reaction, appropriately labeled nucleotides (ex. ^{32}P-α-dCTP) and a DNA polymerase (Klenow fragment) (2, 4)

Note: For nonradioactive detection, colourimetric detection of digoxigenin labeled probes is an alternative. Digoxigenin labeling can be performed using PCR and a digoxigenin-dUTP (5, 6). The digoxigenin labeled probe is detected with anti-digoxigenin-peroxidase conjugate or an alkaline phosphatase conjugate and a colourimetric substrate.

6. Elution of the radioactive probe

6a. By alkali treatment (7)
Wash the Dynabeads once in 50 µl 1x SSC and resuspend in 20 µl 0.15 M NaOH.
Incubate for 10 min at room temperature.
Collect the Dynabeads on the side of the tube and transfer the supernatant with the radioactive labeled probe to a clean tube.
The probe is neutralized by adding 2.2 µl 10x TE buffer, pH 7.5 and 1.3 µl neutralization buffer.
The probe is now ready for use in hybridizations.

6b. By heat treatment (1, 2)

> **Note:** Elution by heat treatment is an alternative method to the alkali method. Heat elution may result in release of small amounts of the complementary biotinylated DNA strand and contamination of the single-stranded probe with double-stranded DNA.

Wash the Dynabeads once in 50 µl 1x SSC and resuspend in another 50 µl 1x SSC.
Incubate at 95°C for 5 min (cover with a lead cap for safety).
Quickly put the tube in the Dynal MPC and transfer the supernatant to a new tube. Be careful when removing the tube from the heating block, there is a risk that the lid of the Eppendorf tube will pop open. Putting the tube directly on ice for two minutes (not longer), decreases this risk.
The supernatant contains the labeled probe ready for use in hybridizations.
The elution procedure may be repeated if the Dynabeads still contain a significant fraction of radioactivity.

10.4 Reference applications

10.4.1 Fingerprinting

The labeled single-stranded DNA probe generated by the above protocol can be used for fingerprinting studies (2, 4). Because of the high sensitivity of the probe, the requirement for blocker DNA is eliminated. The probes give very low unspecific background, since no unlabeled DNA complementary to the probe is present (elution by heat treatment can give a few percent contamination with unlabeled template DNA). This strategy has been used with excellent results.

10.4.2 Northern blot

The method has also been used for Northern blot analysis of seed embryo mRNA (4) and for Northern blot analysis of human mRNA to study the expression of the human repair gene ERCC6 (7).

10.4.3 Solid phase cDNA synthesis

First strand cDNA, synthesized on Dynabeads Oligo(dT)$_{25}$ (see chapter 3), may also be used as the single stranded template in this single stranded probe labeling procedure (8).

10.5 Technical tips

- Wash the Dynabeads M-280 Streptavidin before use to remove the NaN_3 added as a preservative.
- **Use the recommended amount of PCR primer:** Excess PCR primer will compete with biotinylated PCR product and decrease the amount of amplified product bound to Dynabeads M-280 Streptavidin.

- Mix the Dynabeads from time to time during incubation to keep them suspended.
- The non-specific binding of radioactive nucleotides to the Dynabeads can be reduced to a minimum by pre-washing the Dynabeads with a buffer containing 100 mM Na-pyrophosphate before labeling. Subsequently the Dynabeads are washed twice with 100 µl extension buffer before resuspending them in the labeling mixture.

10.6 Buffers and solutions

6x SSC	0.9 M NaCl 0.09 M sodium citrate Dissolve the reagents in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 10 M solution of NaOH. Adjust the volume to 1 litre with H_2O. Sterilize by autoclaving.
1x SSC	0.15 M NaCl 0.015 M sodium citrate Preparation as for 6x SSC.
10x TE buffer	100 mM Tris-HCl, pH 7.5 10 mM EDTA, pH 8.0
Melting solution	0.125 M NaOH 0.1 M NaCl
Neutralization buffer	1.25 M HAc

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11. DETECTION AND QUANTITATION OF AMPLIFIED DNA

11.1 General introduction

Numerous ways are available to analyse PCR amplified material such as gel electrophoresis, hybridization, restriction cleavage and DNA sequencing. DNA sequencing is the most informative of all analysis techniques, however, it is not always needed when only the presence or absence of an amplified fragment has to be confirmed.

The analysis of specific nucleotide sequences using PCR has often overcome the limitations in sensitivity and specificity of conventional DNA detection methods such as probe hybridization techniques.

The DIANA® (Detection of Immobilized Amplified Nucleic Acids) system described in the following sections (protocols in section 11.3 and buffer and solution recipes in section 11.6) is a PCR based solid-phase system especially designed for colorimetric detection of amplified DNA sequences.
The system eliminates the need for electrophoresis, restriction mapping or hybridization assays for identification of PCR products. DIANA is suited for both qualitative and quantitative analysis (1, 2 ,3) using your own custom made PCR primers. The DNA sequence to be detected may be of microbial origin, represent a genetic disorder or an allelic variation. In addition, positive samples can be analyzed by direct solid-phase DNA sequencing according to the protocol in chapter 1 (4, 5).
The DIANA system has been successfully used to detect bacteria (1, 6, 7), virus (8) and parasites (6, 9).

Other approaches to label primers include radioisotopic end labels, fluorescence and digoxigenin (Boehringer Mannheim, Germany).
The QPCR™ System 5000 from Perkin Elmer - Applied Biosystem Division, is based on ECL detection of PCR fragments by magnetic capture using Dynabeads M-450 Streptavidin. The stability of the biotin-streptavidin system is used to separate the two DNA strands with alkali to enable the hybridization with a labeled internal probe carrying a label for chemiluminiscens detection.

11.2 Materials required

- Dynabeads M-280 Streptavidin
- Magnetic Particle Concentrator - Dynal MPC (see Appendix B)
- DIANA buffer
- LacI-β-gal fusion protein
- o-nitrophenyl β-D-galactoside (ONPG)
- Stop solution
- PCR Mastermix
- B&W buffer
- TE buffer
- 0.1 M NaOH
- Qualitative detection:
  Outer primer pair for the first round of PCR (A)
  Inner primer pair for the second round of PCR (B) consisting of
  - one primer biotinylated in the 5' end
  - one primer containing the lac-operator sequence in the 5' end
  The lac-operator sequence is:
  5'-AATTGTTATCCGCTCACAATT-3' (10)
- Quantitative detection:
  Outer primer pair for the first round of PCR
  Inner primer pair (one biotinylated)
  Internal standard: Target DNA containing a section replaced with the lac-operator sequence by *in vitro* mutagenesis.
- Thermocycler
- Test tubes, glassware, pipettes
- Sonicated sperm DNA / Yeast tRNA
- Spectrophotometer / ELISA reader

Note: Buffers and solutions are described in section 11.6.

11.3 Protocols

11.3.1 Qualitative detection

The principle for detection of specific *in vitro* amplified material using Dynabeads M-280 Streptavidin is shown in Figure 11.1. A nested PCR is performed, in which the first step is a standard PCR with outer primers specific for the target DNA sequence. A large number of cycles (i.e. 30-40) is run to guarantee many template molecules for use in the second PCR step. The second PCR is carried out with fewer cycles (i.e. 10-20) and with a primer set annealing to DNA sequences within the DNA fragment amplified in the first PCR. One of the primers in this set is biotinylated while the other contains a non-complementary lac-operator "handle" consisting of 21 nucleotides (this "handle" can be omitted if another detection strategy is used). A successful PCR yields specific DNA with biotin and lac-operator incorporated into the amplified fragment.

In samples not containing the target DNA, nonspecific amplification of random DNA in the first round of PCR may occur (1). Dilution and the use of few cycles in the second round of PCR, results in negligible levels of non-specific fragments containing biotin and lac-operator.

The biotinylated DNA is selectively captured and immobilized onto Dynabeads M-280 Streptavidin and purified by magnetic separation. The suspension is applied to a Dynal MPC to separate the immobilized sequence from the reaction mixture. A fusion protein consisting of the LacI-repressor and β-galactosidase is added to the beads, whereafter the bound enzyme conjugate is detected by adding the chromogenic substrate ONPG. Yellow colored samples are positive and change of absorbance can be measured in an ELISA reader/spectrophotometer.

1. Amplification of the target DNA sequence.
a) First step; non-labeled outer primers: To each PCR tube add the mastermix with the outer primer pair. Add your sample to a final volume of 50 µl. Due to the risk of contamination, the samples should be added in a separate room. Cover each reaction mixture with 50 µl of light mineral oil. Heat the samples (i.e. 94°C for 10 minutes) to denature the target DNA. Run the PCR using 30–40 cycles (a typical program could consist of 94°C, 0.5 min; 50–70°C (depending on primer), 1.0 min; 72°C, 1.0 min.).

b) Second step; one primer biotinylated, one primer containing a non-complementary lac-operator sequence:
To each PCR tube add the mastermix with the inner primer pair. Add 1–5 µl sample from the previous PCR step, and adjust the volume to 50 µl with sterile water. Cover each reaction mixture with mineral oil. Run the second PCR using 10–20 cycles.

2. Immobilization onto Dynabeads M-280 Streptavidin.
Mix 40 µl of washed Dynabeads M-280 Streptavidin (100 µg Dynabeads/40 µl DIANA buffer) with 40 µl of each PCR mixture and incubate for 15 minutes at room temperature. Mix gently during the incubation. Collect the Dynabeads using the appropriate Dynal MPC. Wash once with 100 µl DIANA buffer as described in Appendix B.5.

3. Enzymatic detection.
a) Add 50 µl LacI-β-galactosidase fusion protein and 50 µl DIANA buffer to each tube/well. Incubate for 20 minutes at room temperature. Wash the Dynabeads four times in 150 µl DIANA buffer.

b) Add 50 µl DIANA buffer to each tube/well to resuspend the Dynabeads and then 50 µl ONPG-solution. Positive samples will develop yellow color. After approx. 15 minutes (same for all samples), transfer 75 µl of each reaction mixture to a microtiter tray where each well contains 75 µl stop solution. Measure absorbance at 405 nm in an ELISA plate reader. Alternatively, a spectrophotometer may be used. In this case the Dynabeads are resuspended in 250 µl DIANA buffer, 250 µl ONPG solution is added, and the reaction is stopped using 500 µl stop solution.

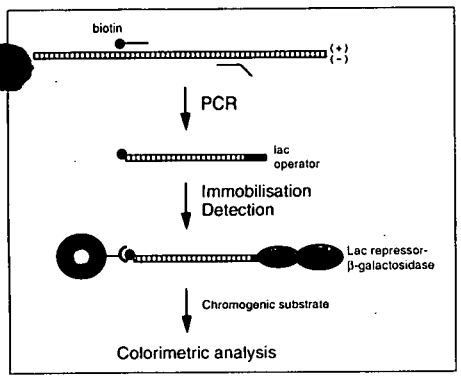

Figure 11.1 The principle for qualitative detection.

11.3.2 Quantitative detection

DIANA also allows quantification of the initial amount of target DNA in a sample, using a competitive approach (3, 11). The principle of the quantitative PCR assay is outlined in Figure 11.2. For each system to be measured, *in vitro* mutagenesis must be performed (12) to introduce the

lac-operator sequence into the middle of a cloned fragment of the target DNA to be analysed (see section 12.2), thus creating an internal standard for the PCR reaction. This internal standard is used as a competitor in the specific assay. A known amount of this competitor fragment is serially diluted. The dilution factor can vary between 1:2 and 1:10, depending on whether accuracy or dynamic range is required. To each of these tubes the same amount of target DNA is added (in the same volume as competitor DNA). **The target DNA must not contain the lac-operator sequence.**
A nested PCR is performed. In the first step non-labeled outer primers are used. In the second step an inner primer pair is used, where one of the primers is biotinylated. The suggested nested PCR procedure allows for all dilution steps of competitor to reach saturation (plateau effect) of PCR.

> **Note:** In quantitative DIANA **none** of the primers contain the lac-operator sequence.

It is assumed that target and competitor DNA are amplified with the same efficiency (for extremely accurate work the amplification efficiency can be tested with known amount of target DNA to obtain the appropriate calibration factor). After PCR, immobilization to Dynabeads M-280 Streptavidin is performed and subsequently fusion protein and substrate (ONPG) are added to all the tubes. The competitor DNA will be detected due to binding of the fusion protein to the lac-operator sequence, and a yellow color will be produced. The colorimetric reaction is stopped after approx. 15 minutes. A high signal as measured in an ELISA reader (spectrophotometer) reflects a high ratio of competitor DNA to target DNA in the sample. In the tubes where target DNA dominates the PCR, no colorimetric reaction will occur, since the lac-operator sequence is absent. A quantification curve is drawn and the activity corresponding to half of maximum activity is calculated, as shown in Figure 11.2. At this activity the initial amount of competitor and target DNA molecules is equal. Thus the amount of sample target molecules can be estimated.

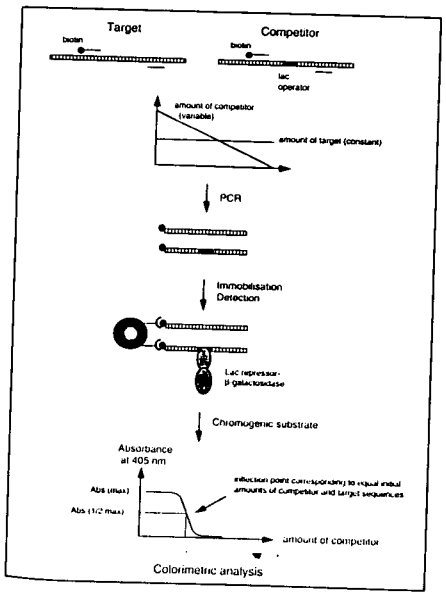

Figure 11.2 The principle of the quantitative PCR detection

1. Construction of competitor DNA.
The lac-operator sequence is introduced into a cloned version of the target DNA sequence by *in vitro* site specific mutagenesis, see section 12.2. The resulting competitor clone is purified and quantified according to standard procedures (Sambrook *et al.* (13)).

2. Co-amplification of target and competitor DNA.
a) First step (outer): Make a serial dilution of the quantified competitor DNA; 5 µl in each step, 6-8 steps in total, dilute in sterile water containing carrier DNA (e.g. yeast tRNA). To each tube/well containing competitor DNA, add 5 µl of the sample and the mastermix with the outer primer pair, and adjust the volume to 50 µl with sterile water. Cover each reaction mixture with [illegible] of mineral oil. Heat the samples to 94°C for 10 minutes to denaturate the DNA. Run the PCR using 30-40 cycles (94°C, 0.5 min; 50-70°C, 1.0 min; 72°C, 1.0 min.). The resulting PCR products are diluted into a new PCR.

b) Second step (inner): To each inner PCR tube or microtiter well, add the mastermix with the inner primer pair and 1-5 µl of the sample from the previous PCR step. Adjust the volume to 50 µl with sterile water and cover each reaction mixture with mineral oil. Run the PCR programme using 10-20 cycles.

3. Immobilization onto Dynabeads.
As described in section 11.3.1, step 2.

4. Enzymatic detection.
As described in section 11.3.1, step 3.

11.3.3 Solid-phase DNA sequencing of positive samples (chapter 1).

Positive samples (generated in qualitative detection) can be treated directly with alkali to remove the bound fusion protein and to melt the double stranded DNA immobilized to the beads. The biotin-streptavidin complex is resistant to the alkali treatment. This treatment produces a single-stranded template suitable for solid-phase DNA sequencing, without the need for cloning procedures or additional template preparation (4, 5).

1. Melting the DNA duplex.
Remove the yellow supernatant by placing the tube/tray in a Dynal MPC. Wash the Dynabeads with the immobilized template twice in 40 µl B&W buffer. Resuspend the Dynabeads in 10 µl 0.1 M NaOH and incubate at room temperature for 5 minutes.

2. Separating the DNA strands.
Place the tube/tray on a Dynal MPC, and transfer the NaOH supernatant (containing the non-biotinylated strand) to a clean tube/well. Wash the Dynabeads once with 50 µl 0.1 M NaOH, once with 40 µl B&W buffer and once with 50 µl TE buffer. Remove the supernatant and adjust the volume with water according to the sequencing protocol to be used.

11.4 Documented DNA detection of microbial pathogens using the DIANA technique.

The DIANA technique has been successfully used for detection of several different microbial pathogens, in different types of samples. In the table 11.1 a summary of pathogens and sample types is shown.

Table 11.1

Type of target	Target	Sample type	Detection method	Ref.
Bacteria	*Chlamydia trachomatis*	Urogenital	Colorimetric/ solid-phase sequencing	1
Parasite	*Plasmodium falciparum*	Blood	Quantitative Colorimetric	3
Parasite	*Plasmodium falciparum*	Blood	Radioactive	7
Virus	Human cytomegalovirus	Urine	Colorimetric/ solid-phase sequencing	8
Parasite	*Plasmodium falciparum*	Blood (fingerprick)	Colorimetric/	9
Bacteria	Shiga-like toxins in *Escherichia coli*	Agar plate cultivation	Radioactive	14
Bacteria	Heat-stabel enterotoxins in *Escherichia coli*	Fecal and intestinal	Colorimetric	15
Bacteria	*Chlamydia trachomatis*	Urine	Colorimetric	16
Parasite	*Plasmodium falciparum*	Blood	Colorimetric	17
Bacteria	*Yersinia enterocolitica*	Seeded food/water	Colorimetric	18
Bacteria	*Chlamydia trachomatis*	Urogenital	Colorimetric	19
Virus	Hepatitis C virus	Blood	Quantitative Colorimetric	20
Virus	Hepatitis C viremia	Blood	Quantitative Colorimetric	21

11.5 Technical tips

- The composition of the PCR mastermix and the parameters of the PCR program may have to be adjusted to your own experimental system. Take great care when moving from first to second round of PCR, to prevent contamination. Use a dedicated area for sample preparation, wear clean disposable laboratory gloves and change the equipment used between each PCR step.

- A positive control should be run to confirm that the system is operating correctly. A negative control (PCR Mastermix containing no DNA) should also be included in the run to control for contamination.

- If problems occur during the enzymatic detection try:
  - In step a) adding 5 µl sonicated sperm DNA (10 mg/ml) to each sample to reduce background signal.
  - In step a) changing tube/well after the last washing step to minimize background signals from fusion protein bound to the wall of the tube/well.

- If the DIANA buffer is exposed to temperatures above 20°C, precipitation of $MgCl_2$ may occur. The $MgCl_2$ may be redissolved by cooling the buffer to 4°C. The precipitation does not affect the buffer's performance in any way.

- The stop solution may precipitate when stored at 4°C. The Na_2CO_3 is redissolved at room temperature. The precipitation does not affect the buffer's performance in any way.

- To achieve an expected titration curve, the binding of LacI repressor-β-gal protein to the immobilized lac operator DNA has to be quantitative (1:1 ratio). Therefore it might be necessary to increase the bead concentration, or to decrease the amount of used PCR product.

11.6 Buffers and solutions

DIANA buffer (20x concentrated)	0.5 M Tris-HCl, pH 8.0 4.0 M NaCl 20 mM EDTA 0.2 M $MgCl_2$ 0.2 M DTT 1% Tween-20
LacI-β-gal fusion protein approx. 2.0 U/ml in:	20 mM Tris-HCl, pH 8.0 300 mM KCl 0.05% Tween-20 10 mM $MgCl_2$ 0.1 M EDTA 10 mM DTT 50% Glycerol
o-nitrophenyl β-D-galactoside (ONPG)	3.75 mg ONPG per tablet One tablet is dissolved in 1.1 ml 1x DIANA buffer.
Stop solution	1.0 M Na_2CO_3, pH 12
PCR Mastermix	5 µl 10x deoxynucleotides (2 mM of each dNTP) 5 µl 10x Tris buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 1% Tween-20) 5 µl 10x $MgCl_2$ (15–120 mM $MgCl_2$) 5 µl of each PCR primer (1 µM) 1 U *Taq* polymerase
B&W buffer	10 mM Tris-HCl, pH 7.5 1.0 mM EDTA 2.0 M NaCl
TE buffer	10 mM Tris-HCL, pH 7.5 1.0 mM EDTA
0.1 M NaOH	

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12. ADDITIONAL APPLICATIONS USING DYNABEADS M-280 STREPTAVIDIN

12.1 SOLID-PHASE GENE ASSEMBLY

12.1.1 General introduction

The use of Dynabeads M-280 Streptavidin as a solid support for immobilization of an oligonucleotide enables a rapid and controlled stepwise hybridization of 5'-phosphorylated oligonucleotides to build up a predesigned gene construct (solid-phase gene assembly) (1-5).

There are several advantages of *de novo* gene design as compared to conventional subcloning of a gene fragment from the natural source:

- optimization of codon usage for improved expression in a heterologous host
- a peptide/protein with known amino acid sequence can be expressed without knowledge of the nucleotide sequence or access to the genetic source
- one or more desired mutations can be introduced in any of the sections
- using multiple alternative oligonucleotides corresponding to a certain region of a protein, designed protein engineering can be performed
- insertion or substitution of a coding region from another gene, or design of new genes to create any chimeric or fusion protein is possible
- introduction of single-cutting restriction sites suitable for further cloning and recombinant DNA technology

Solid-phase gene assembly enables rapid and reliable construction of duplex DNA several hundred base pairs in length and with a low mutation frequency (1). When similar gene assembly methods are carried out in solution, the yield of correct constructs decreases rapidly as the number of oligonucleotides increases. Using automated oligonucleotide synthesis it is still difficult to produce oligonucleotides longer than 100–150 nucleotides with high yield and low error frequency.

A final PCR amplification step, after completed gene assembly enables efficient recovery of gene fragments even if they are present at low concentrations.

12.1.2 Principle of the method

Biotinylation at the 5' end of the first oligonucleotide enables the immobilization on to the Dynabeads M-280 Streptavidin. After the beads with the bound initial oligonucleotide have been washed, the next oligonucleotide (or preannealed linker) is incubated with the Dynabeads in an annealing (or annealing/ligation) buffer at 70°C for 5 minutes, the mixture is allowed to cool to 37°C and then either a washing (2) or a ligation and washing (3, 4) step is performed. The next oligonucleotide (linker) is added and the process is repeated. To end the gene assembly, either anneal a preannealed "final linker" with a blunt end (3, 4) or fill in the 5' overhang with Klenow fragment of DNA polymerase I (2). If no ligation step is included in the protocol during the gene assembly, a ligation step is performed at the end of the gene assembly (2). All oligonucleotides have to be phosphorylated at the 5' end (Figure 12.1).

After completed gene assembly, PCR amplification of the DNA directly from the washed Dynabeads carrying the assembled gene is recommended to increase the amount of assembled DNA (2, 4). Using the initial oligonucleotide or another biotinylated upstream primer enables immobilization of the PCR amplified gene fragment onto Dynabeads M-280 Streptavidin.

Subcloning of the constructed gene fragment can be performed using single cut restriction sites introduced at the start and at the end of the assembled gene (introduced by the first and the last oligonucleotides/linkers) and a suitable vector restricted at the same unique restriction sites (2, 3, 4).

A gap-duplex plasmid formation can also be used for the subcloning of the assembled gene. Then the non biotinylated strand is eluted off with 0.15 M NaOH and neutralized. The eluted single-stranded gene fragment has to carry about 20 nucleotides, both upstream and downstream of the assembled region, complementary to the regions flanking the cloning region in the vector of choice, and can thus form a transformable gap-duplex plasmid when mixed with the single-stranded vector. The transformable gap-duplex plasmid is formed by heating a mixture of the eluted single-stranded gene fragment and the single-stranded vector to 70°C for 5 minutes and then allowing the mixture to cool to room temperature (3).

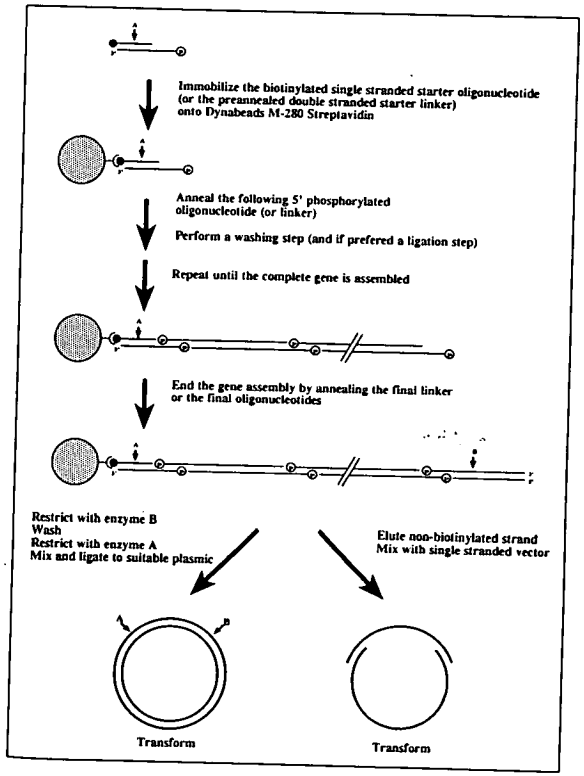

Figure 12.1 The basic principle for solid-phase gene assembly on Dynabeads M-280 Streptavidin.

12.1.3 Technical tips

- A suitable **amount of Dynabeads** to work with is 300 µg (3) in a working volume of 60 µl (3) to 300 µl (2).
- Different **amounts of oligonucleotides** have been tried with good results, 30 pmoles of oligonucleotides per annealing step (3) and 0.2 pmoles of oligonucleotides per annealing step (requires annealing/ligation buffer containing 5% PEG 8000) (4).
- The **length of the oligonucleotides** differs from 33-58 bases in length with 15-18 bases overlaps (3) to 25-34 bases in length and 10-16 bases overlaps (4).
- Using **preannealed linkers** instead of oligonucleotides throughout the gene assembly reduces the number of annealing and washing steps (2).

12.1.4 Buffers and Solutions

Washing/Binding Buffer	1M NaCl 10 mM Tris-HCl pH 75 1 mM EDTA
Annealing/Ligation buffer	50 mM Tris-HCl pH 75 10 mM $MgCl_2$ 1mM ATP 1mM 1,4 dithiothreitol (DTT) 5% PEG 8000

12.1.5 References

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12.2 *IN VITRO* MUTAGENESIS

12.2.1 General introduction

Dynabeads M-280 Streptavidin can be used as a solid support to specifically produce both single-stranded vector DNA and single-stranded template DNA to simplify the performance of oligonucleotide-mediated site specific mutagenesis. The eluted single-stranded vectors and the eluted, mutated, single-stranded fragments can then form gap-duplex plasmids through flanking, complementary double-stranded regions.

The gap-duplex plasmids can be directly transformed into *Escherichia coli* to give high yields (80% is reported (1)) of the specific mutations.

Cloning is achieved without the use of restriction enzymes or ligase and no special vectors or strains are needed. Since the site-specific mutations are produced in single-stranded form, the effect of the mismatch repair system of the host cell is eliminated (1, 2).

12.2.2 Principle of the method

The basic concept is shown schematically in Figure 12.2.

The vector and the fragment to be mutated are immobilized separately on the Dynabeads M-280 Streptavidin through a biotin incorporated into one of the strands.
There are two alternative ways to introduce the biotin into one of the DNA strands (See Appendix E for details about biotinylation procedures):

1. By site-specific restriction, followed by a fill-in reaction using biotin-dNTP's and DNA polymerase, or
2. by PCR amplification using a biotinylated primer

The restriction/fill-in route will yield a biotinylation at the 3' end of the DNA fragment, while the PCR amplification will yield a biotinylation at the 5' end of the DNA fragment.

NOTE: The choice of biotinylation must be carefully considered to ensure that complementary strands are obtained for the vector and insert.

The initial eluted (alkali, 0.15 M NaOH) and neutralized single-stranded vector can be used directly. However, repeated DNA polymerase run-off extensions followed by elution give material for many mutagenesis experiments. The immobilized, single-stranded template vector can be stored at +4°C for several weeks.

For the DNA to be mutated, a single-stranded template suitable for a primer directed polymerase reaction, is obtained by elution of the non-immobilized strand with alkali. The *in vitro* mutagenesis can then either be performed using a double primer system or a single primer system.

In the **double primer system**, a general primer complementary to the vector part of the immobilized fragment is used together with the specific mutagenesis primer with a mismatch in the region to be changed.
An extension reaction using DNA polymerase and DNA ligase is subsequently performed to yield a mutated strand containing the desired mismatch. The synthesized strand is then eluted, neutralized and mixed with the single-stranded vector fragment, which yields a gap-duplex plasmid with overlapping double-stranded regions.

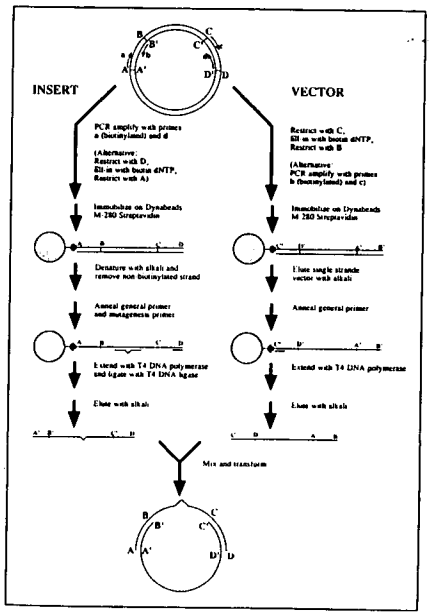

Figure 12.2a A schematic drawing of the basic concept of solid-phase in vitro mutagenesis.

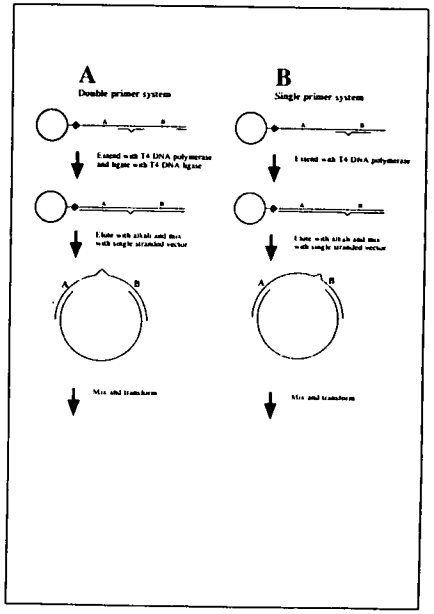

Figure 12.2b A schematic drawing of the *two systems for solid-phase in vitro* mutagenesis using one or two primers.

To be able to use the **single primer system**, the site for the mutation has to be in close proximity to the vector part. Then a combined mutation/extension primer, complementary to both the mismatch region and part of the vector sequence is used. This strategy eliminates the need for the ligation step. A rather short overlapping region will be obtained, but the gap-duplex plasmid can still be used for successful transformation.

E. coli cells are transformed directly with the gap-duplex plasmid and clones are screened by conventional methods. It is good practice to confirm mutagenesis by DNA sequencing (see chapter 1).

A variation of the above method has also been reported (3).
[redacted] amplification is performed in two steps, with a biotinylated M13 universal primer and [redacted]t mutation primer and with a biotinylated M13 reverse primer and a second mutation primer, respectively. The PCR products contain overlapping regions at the site of the mutagenesis primers and after immobilization and purification of the biotinylated single-stranded fragments, the fragments are mixed, annealed and extended under PCR conditions but in absence of primers.
The double-stranded mutated fragment can be cleaved within flanking polylinker regions and be subcloned and expressed.
By using biotinylated M13 universal and reverse primers, the method may be used with any target sequence cloned in pUC plasmids, M13 phage or their derivatives.
Hall and Emery (3) have validated this method using a 4kb fragment.

12.2.3 Technical tips

Dynal recommends producing single-stranded vector by the restriction/fill-in procedure (alternative 1 under section 12.2.2). A non-PCR approach has the advantage, that the accumulation of polymerase errors whenever PCR products are cloned, is avoided (4).

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12.3 DIRECT CLONING

12.3.1 General introduction

Cloning of PCR products is often a difficult and tedious process. The use of Dynabeads M-280 Streptavidin, as a magnetic solid-phase, simplifies the production of single-stranded DNA that can be used for direct cloning.
The DNA fragment (genomic or of other source) that one wishes to clone is PCR amplified, made single-stranded and mixed with single-stranded vector to form a transformable gap-duplex plasmid.
There is no need for restriction enzymes, linkers or ligase and yields of more than 90% of the desired recombinant molecule are achieved (1). This strategy in combination with direct solid-phase DNA sequencing (2), has been used to analyse individual alleles in the human apolipoprotein E locus (1).

12.3.2 Principle of the method

As with any cloning protocol, two preparations are required - the cloning vector and the insert.

Both the vector and the fragment to be cloned, have to be immobilized on the Dynabeads M-280 Streptavidin through a biotin incorporated into one of the strands. (See Appendix E for details about biotinylation procedures).

Dynal recommends biotinylating the vector DNA using a classic fill-in reaction. This is performed by a site-specific restriction, followed by a fill-in reaction using biotin-dNTP's and DNA polymerase.

PCR amplify the insert using a nested primer procedure. The internal primers are designed with handle sequences complementary to the ends of the single-stranded vector. One of the primers is biotinylated to introduce the biotinylation into the insert DNA.

> **Note:** The non-biotinylated insert strand is the strand that is eluted and the ends of which have to be complementary to the ends of the eluted single-stranded vector.

The biotinylated vector DNA and the PCR amplified biotinylated insert DNA are immobilized separately onto Dynabeads M-280 Streptavidin.

The non-biotinylated vector strand and the non-biotinylated insert strand are eluted with alkali, neutralized and mixed to form gap-duplex recombinant plasmids, see Figure 12.3.

E. coli cells are transformed directly with the gap-duplex plasmid and clones are screened by conventional methods. It is good practice to confirm each clone by DNA sequencing (see chapter 1).

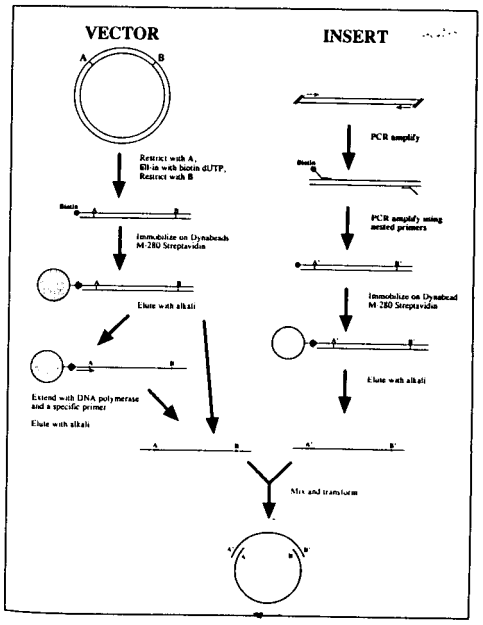


Figure 12.3 The basic concept for solid-phase cloning using magnetic separation of DNA fragments.

12.3.3 Technical tips

Dynal recommends producing single-stranded vector by the restriction/fill-in procedure (as described under section 12.3.2). A non-PCR approach has the advantage, that the accumulation of polymerase errors whenever PCR products are cloned, is avoided (3).
All PCR cloned DNA should be confirmed by sequencing.

12.3.4 References

1. Hornes E, Hultman T, Moks T, Uhlén M
   Direct Cloning of the Human Genomic Apolipoprotein B Gene Using Magnetic Separation of Single-Stranded DNA.
   BioTechniques 1990;9(6)730-737 (Ref. Number: 458)
2. Hultman T, Ståhl S, Hornes E, Uhlén M
   Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support.
   Nucleic Acids Research 1989;17(13)4937-4946
   (Ref. Number: 123)
3. Tindall K.R, Kunkel T.A
   Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase.
   Biochemistry 1988;27:6008-6013

12.4 LIGATION-MEDIATED PCR: PRE-ENRICHMENT OF GENE SPECIFIC FRAGMENTS AND CAPTURE OF AMPLIFIED PRODUCT

12.4.1 General introduction

Ligation-mediated PCR (LM-PCR) can be used for *in vivo* footprinting (1) and genomic sequencing (2). To increase the specificity of the method the Dynabeads M-280 Streptavidin can be used to pre-enrich gene specific fragments and to capture amplified products.
Ligation-mediated PCR is a single-sided PCR technique, which combines the specificity of hybridizing to a single primer site with (non-specific) blunt end linker ligation, to amplify and identify sequences where only one side is known.
Chromosomal DNA is nicked or cleaved in a defined way, the DNA denatured and a gene specific primer extended with a DNA polymerase to generate a blunt end. A linker is ligated to the newly synthesized blunt end. A second gene specific primer and a linker primer are then used to specifically amplify the fragments by PCR. Either the specific primer or the linker, can be biotinylated to enable immobilization on Dynabeads M-280 Streptavidin. Products are identified by Southern blotting or by extension with a third labeled primer. Fragments are analysed on a polyacrylamide sequencing gel.

12.4.2 Principle of the method

Pre-enrichment of specific fragments.
Using a **biotinylated gene specific primer** for the first extension of the target DNA, enables enrichment of the extension product prior to the PCR amplification by immobilization onto Dynabeads M-280 Streptavidin (Figure 12.4) (3). This extension product capture reduces the complexity of the DNA in the PCR amplification. Amplification of non-specific sequences is thereby prevented and the PCR reactions are expected to be more efficient when done on only a small amount of DNA that is already enriched in specific target sequences.

Capture of amplified product.
In the „Linker Tag Selection" method a **biotinylated linker oligonucleotide** is used in the ligation and PCR amplification steps (4, 5). The PCR product is immobilized onto Dynabeads M-280 Streptavidin and the second gene specific primer, along with other PCR reaction contaminants can be efficiently washed away and the non-biotinylated strand can be eluted. Efficient labeling of amplified DNA can be performed with a third labeled primer. Immobilization of the PCR product onto Dynabeads M-280 Streptavidin improves the labeling reaction both quantitatively (removal of competing second gene specific primer and removal of the complementary strand by alkali elution) and qualitatively (the design of the labeling primer is not so critical when it does not have to compete with the second PCR primer) (4, 5).

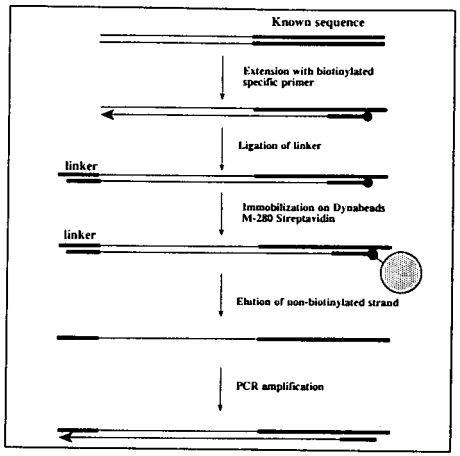


Figure 12.4 The principle for pre-enrichment of gene specific DNA fragments in ligation-mediated PCR.

Isolation of chromosomal DNA fragments
With a combination of a branch-capture reaction and a ligation-mediated PCR amplification any chromosomal DNA can be cloned.
In a branch-capture reaction (BCR), a displacer strand is used to bring a complementary recipient duplex strand 3' end (digested chromosomal DNA) adjacent to the 5' end of a complementary linker strand, creating a substrate for covalent linkage by ligase (6). Extension of the displacer strand with DNA polymerase generates fragments with a blunt end suitable for ligation of an unphosphorylated second linker. The oligonucleotide complementary to the displacer strand of the **displacer linker is biotinylated**, and the extended DNA fragment with the ligated second linker is immobilized onto Dynabeads M-280 Streptavidin. Excess primer, chromosomal DNA and enzymes can be conveniently removed and the complementary non-biotinylated strand can be eluted. A second specific primer 3' to the displacer primer, together with an oligonucleotide of the second linker is used for the PCR amplification of the desired fragment (6).

12.4.3 References

1. Mueller P.R, Wold B
   In vivo footprinting of muscle specific enhancer by ligation mediated PCR.
   Science 1989;246:780-786
2. Pfeifer G.P, Steigerwald S.D, Mueller P.R, Wold B, Riggs A.D
   Genomic sequencing and methylation analysis by ligation mediated PCR.
   Science 1989;246:810-813
3. Törmänen V.T, Swiderski P.M, Kaplan B.E, Pfeifer G.P, Riggs A.D
   Extension product capture improves genomic sequencing and DNase I footprinting by ligation-mediated PCR.
   Nucleic Acids Research 1992;20:5487-5488
   (Ref. Number: 833)
4. Quivy J-P, Becker P.B
   An improved protocol for genomic sequencing and footprinting by ligation mediated PCR.
   Nucleic Acids Research 1993;21:2779-2781
   (Ref. Number: 1028)
5. Quivy J-P, Becker P.B
   Direct Dideoxy Sequencing of Genomic DNA by Ligation-Mediated PCR.
   BioTechniques 1994;16:239-241 (Ref. Number: 1113)
6. Barany F
   The Ligase Chain reaction in a PCR World.
   PCR Methods and Applications 1991;1:5-16
   (Ref. Number: 577)

12.5 CAPTURE OF LARGE INSERTS AND RESTRICTION DIGESTS ON DYNABEADS

12.5.1 General introduction

Genomic libraries in Lambda vectors can be easily constructed, stored and handled, but a major drawback is the difficulty to separate the vector sequence from the insert. To be able to perform shotgun sequencing or genomic walking, the Lambda arms have to be separated from the insert.

12.5.2 Principle of the method

One method to overcome this problem is described by Elgar and Brenner (1). They make use of the cohesive ends of Lambda to biotinylate the DNA by a fill-in reaction using Klenow polymerase in combination with one or two biotin-nucleotides. Any annealed cohesive ends are melted before the fill in reaction of the overhangs. After the incorporation, the DNA is bound to Dynabeads M-280 Streptavidin. The bound Lambda phage DNA is collected with a Dynal MPC, washed, resuspended in an appropriate restriction enzyme buffer and the DNA is cleaved using an enzyme that cuts in the phage polylinker but nowhere else in the vector arms, see Figure 12.5. The beads with the attached vector arms are separated from the insert by magnetic capture.
The released DNA can be used directly for shotgunning after shearing or digestion with a frequent cutter.

Sanford and Elgar (2) describe a modification of this method. The cohesive ends are biotinylated as described above, but the DNA is cut with an enzyme which has no sites in the vector arms or the polylinker. This liberates internal fragments of the insert and leaves the end fragments attached to the arms. The Lambda arms with the end fragments are captured on Dynabeads M-280 Streptavidin, washed and then the end fragments are released by digestion with a restriction enzyme cutting the polylinker. These fragments can be cloned into a plasmid vector or be radiolabeled after separation by agarose gel electrophoresis, see Figure 12.5.
This method allows directional walking from a Lambda clone without first having to obtain a detailed restriction map.

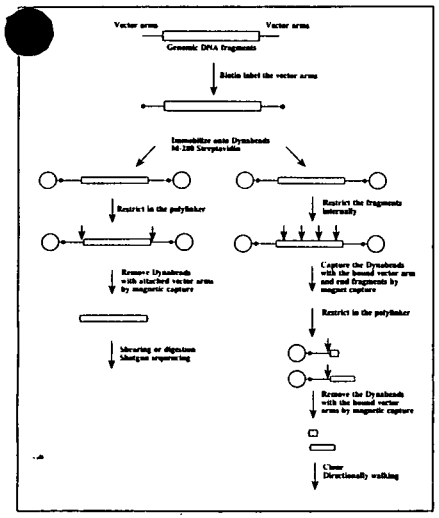


Figure 12.5 A schematic illustration of the principle of capture of large inserts and digests on Dynabeads. Note that in many cases one fragment can bind both ends to the same bead.

12.5.3 References

1. Elgar G.S, Brenner S
   A novel method for isolation of large insert DNA from recombinant lambda DNA.
   Nucleic Acids Research 1992;20:4667
   (Ref. Number: 835)
2. Sanford R.N, Elgar G.S
   A novel method for rapid genomic walking using lambda vectors.
   Nucleic Acids Research 1992,20:4665-4666
   (Ref. Number: 834)

A. DYNABEADS M-280 - PHYSICAL CHARACTERISTICS

Dynabeads M-280 are uniform, monodisperse, superparamagnetic, polystyrene beads.

Diameter: 2.8 μm +/- 0.2 μm (C:V max 3%)
Surface area: 5-8 m^2/g
Specific gravity: approx. 1.3 g/cm^3
Iron content: approx. 13%
Magnetic mass susceptibility: 100 +/- 25×10^{-6} m^3/kg

B. MAGNETIC PARTICLE CONCENTRATORS (DYNAL MPCs)

B.1 General introduction

Dynal Magnetic Particle Concentrators (Dynal MPC) are made from neodymium-iron-boron permanent magnets embedded in disinfectant-proof material. The Dynal MPC guarantees satisfactory isolation of Dynabeads.

B.2 Products for molecular biology (small volumes)

Dynal MPC-E (Prod. No. 120.04)
Dynal MPC for isolation of Dynabeads in molecular biology applications. The rack is designed to hold up to **six tubes of Eppendorf® type** with 8–10 mm diameter and with an optimal working volume up to 0.5 ml.

Dynal MPC-E-1 (Prod. No. 120.07)
Like Dynal MPC-E, for **one tube only.**

Dynal MPC-M (Prod. No. 120.09)
Dynal MPC equipped with a removable magnet back plate for isolation of Dynabeads in molecular biology and microbiology applications. The rack is designed to hold up to **ten tubes of Eppendorf type** with 8–10 mm diameter and with an optimal working volume of 0.2–1.2 ml.

Dynal MPC-96 (Prod. No. 120.05)
Dynal MPC designed for isolation of Dynabeads in molecular biology applications in different types of **flexible 96-well microtiter plates.** Optimal working volumes of the Dynal MPC-96 are up to 50 µl for PCR and sequencing reactions and 100 µl for biomagnetic separation.

Dynal MPC-9600 (Prod. No. 120.06)
Dynal MPC designed for isolation of Dynabeads in molecular biology applications in up to **96 reaction tubes (0.2 ml thinwall PCR tubes)** or up to 48 reaction tubes (0.5 ml) in the Perkin Elmer GeneAmp® PCR System 9600. The optimal working volume of the Dynal MPC-9600 is from 5 to 200 µl.

Dynal MPC-P-12 (Prod. No. 120.10)
Dynal MPC for isolation of Dynabeads in molecular biology. The magnet is designed to hold up to **twelve standard 0.5 ml PCR microtubes** in two rows of six. It has a removable central magnet to facilitate easy washing of Dynabeads with high and low positions for optimum separations in large or small volumes and allows the Dynabeads pellet to be concentrated down the tube wall. Each tube position is numbered consecutively to avoid sample mix up. The optimal working volume of the Dynal MPC-P-12 is from 5 to 200 µl.

B.3 Products for larger sample sizes

Dynal MPC-1 (Prod. No. 120.01)
Dynal MPC for isolation of Dynabeads in **single tubes of variable diameters** (10–30 mm) and volumes (5–50 ml).

Dynal MPC-6 (Prod. No. 120.02)
Dynal MPC for isolation of Dynabeads simultaneously in **six tubes with diameters 10–15 mm** and volumes 5–15 ml.

B.4 Principle

Magnetic isolation with the Dynal MPCs is based on the principle that the analyte (macromolecules such as DNA/RNA-fragments, virus or bacteria) to be isolated are specifically bound to the Dynabeads. The bound analyte can easily and specifically be removed from a heterogeneous mixture by inserting the test tubes containing the target material and the Dynabeads into the Dynal MPCs.

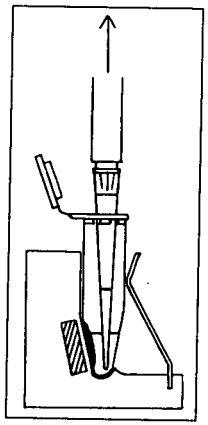
The Dynabeads as well as the bound analyte will be attracted and kept in place on the wall of the test tubes by the magnetic field. After washing, the specific analyte bound to the Dynabeads can be used for further studies (positive selection).

The material remaining in the supernatant, depleted of the analyte specifically bound to the Dynabeads, may also be used for further studies (negative selection).

For detailed information, please see each product insert.

B.5 Instructions for use

B.5.1 Magnetic separation



1. The test tube(s)/microtiter plate(s) containing the mixture to be examined and the appropriate Dynabeads are mixed and incubated according to the desired experimental protocol.
2. For separation, place the test tube(s)/microtiter plate(s) in the Dynal MPC. Ensure that the tube(s)/microtiter plate(s) are properly inserted. Do not move the tube(s)/microtiter plate(s) during the separation process.
3. Allow the tube(s)/microtiter plate(s) to remain in the Dynal MPC for 1–2 minutes. During this time the Dynabeads with the specifically bound target material are attracted to the wall of the test tube by the magnetic field.
4. Remove the supernatant by aspiration with a pipette while the tube(s)/microtiter plate(s) remain(s) in the Dynal MPC (see figure to the left). The target material specifically bound to Dynabeads are held on the wall of the microtubes by the magnetic field. **Avoid aspiration along the wall where the Dynabeads are attracted by the magnet.**

B.5.2 Washing of the target material

1. After discarding the supernatant (step 4 above), remove the test tube(s) from the Dynal MPC. Add the washing solution along the wall of the test tube(s)/microtiter plate(s) where the target material specifically bound to the Dynabeads are held. Resuspend the isolated analyte bound to the Dynabeads gently. Insert the tube(s)/microtiter plate(s) into the Dynal MPC for a minimum of one minute between each washing cycle according to the experimental protocol.
2. After discarding the final washing solution, the analyte is resuspended in an appropriate medium by flushing the desired volume of medium along the side of the tube where the target material is held.
3. The material immobilized on the Dynabeads is now ready for further studies.

B.6 Disinfection

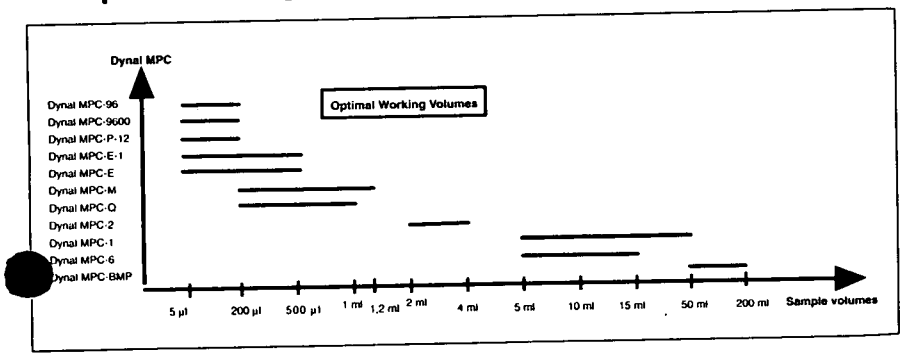
The Dynal MPCs can be washed in a mild soap solution and hot water. If disinfection is necessary, use 70% ethanol.

Note: Do not autoclave the Dynal MPCs.

Note: Magnetic isolation is, as all biological techniques, critically dependent on the specificity and the avidity of the ligands used with the Dynabeads. The Dynal MPCs only guarantee a satisfactory isolation of Dynabeads, not the isolation of a certain macromolecule, antigen, cell or analyte.

Note: The Dynal MPCs should not be kept in close contact with magnetic tapes, computer discs or other magnetic storage systems, as these can be damaged by the strong magnetic field.

B.7 Optimal working volumes of the Dynal MPCs



C. DESCRIPTION OF DYNAL MOLECULAR BIOLOGY PRODUCTS

C.1 General introduction

Solid-phase methods have proven very useful for the separation, synthesis and detection of biomolecules. With the solid-phase approach, high yield and reproducibility can be obtained and automation is facilitated since reaction buffers can be rapidly and easily changed.

The use of Dynabeads, monosized superparamagnetic beads, combines the convenience of magnetic separation with reaction kinetics similar to those found in free solution.

C.2 Major advantages of using Dynabeads M-280 products

The uniformity of the particles and their precisely defined size ensures that each bead has identical chemical and physical properties, hence every milligram of Dynabeads contains exactly the same number of beads. All Dynabeads will perform identically in suspension with respect to sedimentation, magnetic attraction and kinetics of binding to other molecules.
The monodisperse characteristics of the Dynabeads ensures that each bead has the same surface activity.
Each bead contains the same amount of magnetic material and respond equally to the effect of the magnetic field.
The Dynabeads are superparamagnetic and have no magnetic remanence when removed from the magnetic field. This is of importance for the application of the Dynabeads. They can be magnetically collected and resuspended any number of times. Any residual magnetism would seriously impair redispersion of the Dynabeads.
The surface of the Dynabeads M-280 has been optimized for use in enzymatic reactions.

C.3 Dynabeads M-280 Streptavidin

Description:
Dynabeads M-280 Streptavidin are monodisperse magnetic beads coated with purified streptavidin. Streptavidin is a protein with the approximately MW of 66 kDa (1) and consists of four identical subunits, each having the same high binding affinity for biotin. It has the same biotin binding affinity as avidin, but less non-specific binding affinity is observed (2).

Concentration:
Dynabeads M-280 Streptavidin are supplied as a suspension containing 6.7 x 10^8 Dynabeads/ml (10 mg/ml), dissolved in phosphate buffered saline (PBS) pH 7.4, containing 0.1% BSA and 0.02% NaN_3 as a preservative.

Binding capacity:
See "Immobilization of biotinylated nucleic acids", appendix F for capacity of the Dynabeads M-280 Streptavidin.

Storage:
Dynabeads M-280 Streptavidin should be stored at 2 - 8°C.
The Dynabeads may be frozen in the buffer they are supplied in, but freezing is not recommended.

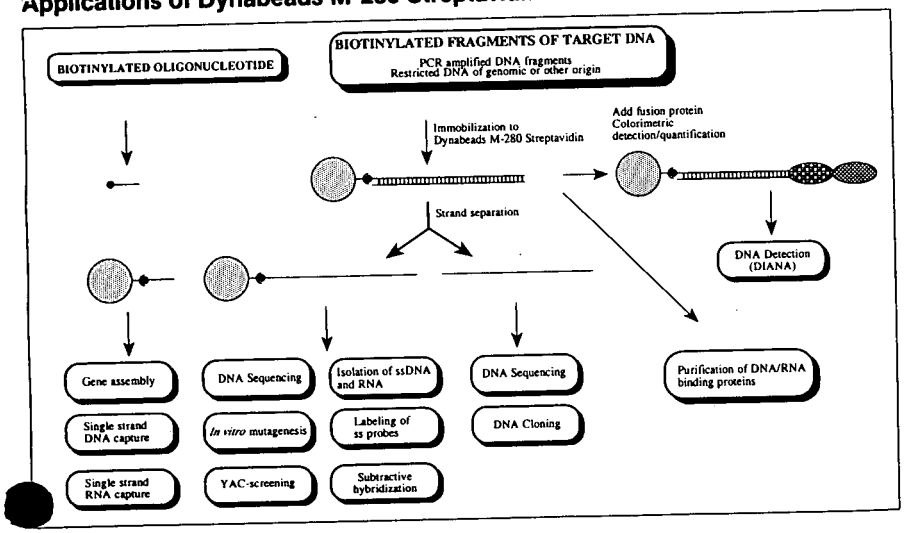
Note: Repeated freezing and thawing should be avoided. Do not store or freeze the Dynabeads in distilled water. Keep the vials of Dynabeads in an upright position to ensure the Dynabeads are covered with buffer, as drying will reduce their performance.

Applications:
Dynabeads M-280 Streptavidin can be used for direct immobilization of biotinylated compounds such as double-stranded or single-stranded DNA, RNA, proteins, sugars and lectins.

Applications include: solid-phase DNA sequencing, DNA/RNA capture by hybridization, labeling of single-stranded nucleic acid probes, gene assembly, *in vitro* mutagenesis, YAC-screening, DNA cloning, sequence-specific purification of DNA/RNA binding proteins, cell separation and bacteria isolation.

The stability and strength of the biotin-streptavidin interaction (K_d=10^{-15} M) allows DNA manipulations, such as strand melting, elution and hybridization (using alkali, temperature or formamide) to be performed without interfering with the immobilization of the DNA on the beads.

Applications of Dynabeads M-280 Streptavidin



Products available:		
Dynabeads Template Preparation Starter Kit For preparation of single-stranded templates of PCR products for **direct sequencing of cloned DNA.** Includes Dynabeads M-280 Streptavidin (1x0.4 ml), primer set A, Binding and Washing buffer and a Dynal MPC-E-1 (Magnetic Particle Concentrator holding a single Eppendorf tube).		**(Prod. no. 612.02)**
Dynabeads Template Preparation Kit For preparation of single-stranded templates of PCR products for **direct sequencing of cloned DNA.** Includes Dynabeads M-280 Streptavidin (2x1 ml), primer set A, primer set B and Binding and Washing buffer.		**(Prod. no. 612.01)**
Dynabeads M-280 Streptavidin	(2x1 ml)	**(Prod. no. 112.05)**
Dynabeads M-280 Streptavidin	(2x5 ml)	**(Prod. no. 112.06)**

C.4 Dynabeads Oligo $(dT)_{25}$

Description:
Dynabeads Oligo $(dT)_{25}$ contain 25 nucleotides long chains of deoxythymidines covalently attached to the bead surface via a 5' linker group.

Concentration:
Dynabeads Oligo $(dT)_{25}$ are supplied as a suspension of 3.3 x 10^8 beads per ml (5 mg/ml) in phosphate-buffered saline (PBS) pH 7.4, containing 0.02% NaN_3 as a preservative.

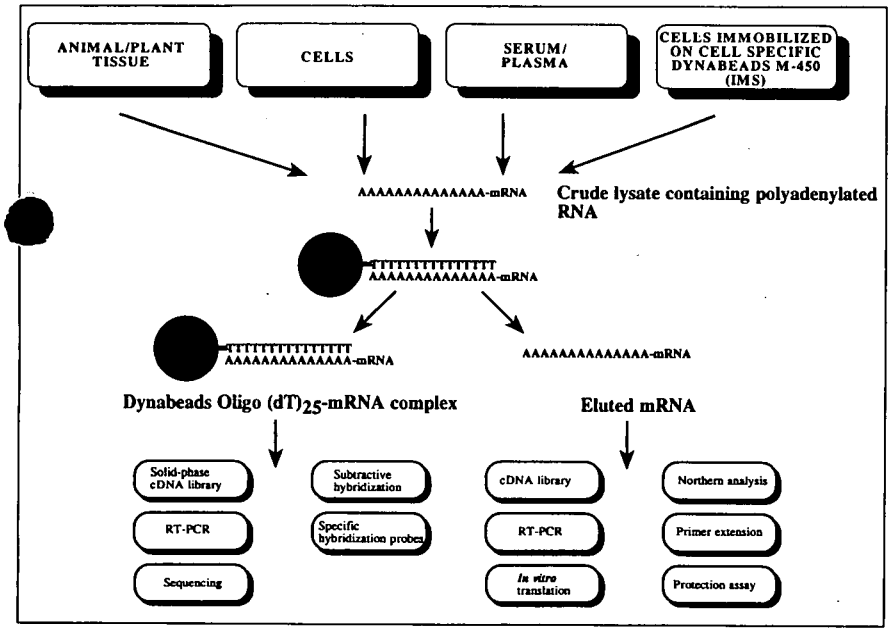
Binding capacity:
Up to 2.5 µg poly(A)$^+$ mRNA may be isolated per mg Dynabeads depending on the tissue or cell type and the expression level of the mRNA. 1 ml Dynabeads Oligo $(dT)_{25}$ (5 mg) may therefore be used to isolate between 6 µg and 12 µg of poly(A)$^+$ mRNA depending on tissue or cell type. If the same Dynabeads are reused for a total of 5 mRNA isolations (four regeneration cycles), the total capacity of 1 ml Dynabeads is between 30 µg and 60 µg of mRNA.
For regeneration of Dynabeads Oligo $(dT)_{25}$, see section 2.4.

Storage:
The Dynabeads Oligo $(dT)_{25}$ should be stored at 2 - 8° C.
The Dynabeads may be frozen in the buffer they are supplied in, but freezing is not recommended.

Note: Repeated freezing and thawing should be avoided. Do not store or freeze the Dynabeads in distilled water. Keep the vials of Dynabeads in an upright position to ensure the Dynabeads are covered with buffer, as drying will reduce their performance.

Applications:
Dynabeads Oligo $(dT)_{25}$ are designed for rapid isolation of highly purified, intact poly (A)$^+$ mRNA from eukaryotic total RNA or directly from crude extracts of animal tissue, cells and plants. The direct mRNA isolation is performed in 15 minutes without having to perform any previous purification steps. All common buffers for mRNA purification and isolation can be used with Dynabeads Oligo $(dT)_{25}$. However Dynal recommends the use of the buffers described in section 2.6 to take full advantage of the unique properties of the Dynabeads. The isolated mRNA is in a concentrated form and may be used directly, in all downstream applications in molecular biology including enzymatic applications: solid-phase cDNA library, RT-PCR, S1 nuclease analysis, ribonuclease protection, primer extension, dot and slot hybridization, *in vitro* translation experiments, subtractive hybridization and Northern blotting. The isolated mRNA is ideal for gene cloning and gene expression analysis.

Applications of Dynabeads Oligo $(dT)_{25}$



Products available:		
Dynabeads mRNA DIRECT kit For direct mRNA isolation from **animal tissues, cells and plants.** Includes 1 ml Dynabeads Oligo $(dT)_{25}$, suitable extraction-, washing- and elution buffers, and reagents for efficient regeneration of the Dynabeads. Note: Magnetic Particle Concentrator is not included.		**(Prod. no. 610.11)**
Dynabeads mRNA purification kit For purification of mRNA from **total RNA.** The kit includes 1 ml Dynabeads Oligo $(dT)_{25}$, binding-, washing- and elution buffers and a Dynal MPC-E-1 (Magnetic Particle Concentrator holding a single Eppendorf tube).		**(Prod. no. 610.01)**
Dynabeads Oligo $(dT)_{25}$	(2 x 1 ml)	**(Prod. no. 610.02)**
Dynabeads Oligo $(dT)_{25}$	(5 x 1 ml)	**(Prod. no. 610.05)**

C.5 Dynabeads lacZ

Description:
Dynabeads lacZ have a biotinylated oligonucleotide complementary to a part of the lacZ-region in different vectors, linked to the surface of Dynabeads M-280 Streptavidin. The oligonucleotide is 40 nucleotides long with the following sequence:

5'biotin-TT ATC CGC TCA CAA TTC CAC ACA ACA TAC GAG CCG GAA GC-3' and is complementary to the (+) strand of e.g. M13 vectors. For further details see section 6.4 (M13mp18 information).

Concentration:
Dynabeads lacZ are supplied as a suspension containing 6.7 x 10^8 Dynabeads/ml (10 mg/ml), in a storage buffer containing 250 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.1% Tween-20 and 0.02% NaN_3 as a preservative.

Binding capacity:
The yield should be at least 1 µg DNA template/mg Dynabeads lacZ.
The Dynabeads lacZ can be reused four times. For regeneration of Dynabeads lacZ, see section 6.3.4.

Storage:
The Dynabeads lacZ should be stored at 2 - 8°C.
The Dynabeads may be frozen in the buffer they are supplied in, but freezing is not recommended.

Note: Repeated freezing and thawing should be avoided. Do not store or freeze the Dynabeads in distilled water. Keep the vials of Dynabeads in an upright position to ensure the Dynabeads are covered with buffer, as drying will reduce their performance.

Application:
Dynabeads lacZ are used to purify single-stranded templates for DNA sequencing from vectors containing the lacZ region, such as M13 and M13 derived vectors. LacZ containing vectors are purified through hybridization with the complementary oligonucleotide linked to the Dynabeads.

Products available:		
Dynabeads lacZ ssM13 Purification Kit For preparation of single-stranded templates for DNA sequencing from vectors containing the lacZ region, such as M13 and M13 derived vectors. Includes Dynabeads lacZ (2x1.3 ml), lysis buffer, hybridization solution, 3x washing buffer, elution buffer, 3x recondition solution, storage buffer and control template.		**(Prod. no. 625.01)**
Dynabeads lacZ	(10 ml)	**(Prod. no. 625.06)**

C.6 References

1. Argaraña CE, Kuntz ID, Birken S, Axel R, Cantor CR
Molecular cloning and nucleotide sequence of the streptavidin gene
Nucleic Acids Research 1986;14(4):1871-1882

2. Pähler A, Hendrickson WA, Gawinowicz Kolks MA, Argaraña CE, Cantor CR
Characterization and crystallization of core streptavidin
J Biol Chem 1987;262(29):13933-13937

D. PREPARATION OF DYNABEADS M-280 PRODUCTS

D.1 General preparation of supplied Dynabeads M-280 products

Dynabeads M-280 products should be washed before use to remove the 0.02% NaN_3 added as a preservative. The Dynabeads may be washed in bulk. The washing procedure is facilitated by using a Dynal MPC.
Be sure to work under RNase-free conditions when preparing Dynabeads Oligo $(dT)_{25}$.

D.1.1 Protocol

1. Resuspend the Dynabeads M-280 products by gently shaking the vial to obtain a homogeneous suspension.
2. Add the appropriate amount of Dynabeads M-280 product to a tube.
3. Place the tube in the Dynal MPC for 1-2 min. Do not move the tube during the separation process.
4. Remove the supernatant by aspiration with a pipette while the tube remains in the Dynal MPC. Avoid touching the inside wall of the tube, where the Dynabeads are attracted to the magnet, with the pipette tip.
5. Remove the tube from the Dynal MPC. Add the recommended buffer along the inside of the tube where the Dynabeads are held. Use the same volume as in step 2 and resuspend gently.
6. Repeat steps 4 and 5.
7. Again remove the tube from the Dynal MPC and add a suitable volume of the recommended buffer to obtain an appropriate working concentration of Dynabeads.

D.2 Preparation of Dynabeads M-280 Streptavidin for RNA manipulations

Note: Dynabeads M-280 Streptavidin is NOT supplied in RNase-free solution.

D.2.1 Protocol

1. Add DEPC to a final concentration of 0.1% (1 ml/l) to solution A and solution B.
2. Shake vigorously.
3. Incubate at room temperature for 1 hour and autoclave the solutions.
4. Wash the Dynabeads twice with the same volume of solution A for 1-3 min.
5. Wash the Dynabeads once with the same volume of solution B.
6. Finally, resuspend the Dynabeads in solution B.

D.3 Buffers and solutions

Solution A	DEPC-treated 0.1 M NaOH 0.05 M NaCl
Solution B	DEPC-treated 0.1 M NaCl

E. BIOTINYLATION PROCEDURES

E.1 General introduction

Biotinylated nucleic acids and proteins are easily bound to Dynabeads M-280 Streptavidin. Following the binding procedure, the biotinylated product is easily maneuvered due to the unique magnetic properties of the Dynabeads M-280 Streptavidin and the strength and stability of the biotin/streptavidin linking system (K_d=10^{-15} M). Because of simplified handling, experimental procedures, such as changing buffers, are easily accomplished.

Notes:
- All biotin reagents should contain a spacer arm, at least 6 C-atoms in length, to reduce steric hindrance.
- Free biotin in your sample will reduce the binding capacity of the Dynabeads M-280 Streptavidin. A disposable column packed with Sephadex® (Pharmacia Biotech AB, Uppsala, Sweden) will remove unincorporated biotin.
- Biotinylated oligonucleotide primers should be purified by reverse phase HPLC/FPLC chromatography for optimal binding efficiency.
- Specific biotinylation in the 5'-end is recommended to maintain the 3'-end free for elongation.

E.2 Biotinylation of nucleic acids

E.2.1 Oligonucleotide primers

Many protocols now exist for biotinylation of oligonucleotide primers to be used for the amplification of a target gene.

Direct incorporation of biotin at the 5' end of the oligo during DNA synthesis using biotin phosphoramidite is recommended (1, 2, 3). The use of biotin phosphoramidite in the synthesis reaction allows biotinylation at the 5' end or any internal position (single or multiple) with no effect on the specificity or hybridization temperature of the labeled oligonucleotide.
Biotin phosphoramidites are commercially available from a number of companies and biotinylated oligonucleotides can be obtained from several commercial sources offering oligonucleotide synthesis services. Ask for the HPLC/FPLC data belonging to the specific biotinylated primer you ordered so that you can control the quality.

Recommended biotin phosphoramidite:
- Cambridge Research Biochemicals Ltd., Cheshire, UK
  DMT-BIOTIN-C6-PA, Cat. No. DR-16-100A/B
- Pharmacia Biotech AB, Uppsala, Sweden
  Biodite™ biotin amidite, Code No. 27-1793-01
- Amersham International plc, Buckinghamshire, UK
  Biotin phosphoramidite, RPN 2012
- Peninsula Laboratories, Inc., Belmont, CA, USA
  5'-Biotin-C_{16}-CEP, Cat. No. N4038
- Clontech Laboratories, Inc., Palo Alto, CA, USA
  Biotin-ON™ Phosphoramidite, Cat. No. 5191-1/2/3
- Cruachem Ltd., Glasgow, Scotland
  Biotin-CE Phosphoramidite, Cat. No. 22-0001-35/17
- Perkin Elmer - Applied Biosystems Division, Foster City, CA, USA
  Biotin Amidite, Cat. No. 401395/6

Alternatively, the USB™ Oligonucleotide Biotin Labeling Kit (Product No. 72350, United States Biochemical Corporation, Cleveland, OH, USA) can be used for efficient labeling of standard, deblocked oligonucleotides with a single biotin residue at the 5' end. The method requires two steps and the resulting biotinylated oligonucleotide is then purified using one of several methods.

If desirable, it is also possible to directly biotinylate your own oligonucleotide primer at the 3' end by a 3'-labeled polymer support designed for use in an automated DNA synthesizer (4).

Recommended biotin-CPG:
- Clontech Laboratories, Inc., Palo Alto, CA, USA
  3' Biotin-ON CPG pre-packed columns, Cat. No. 5225-1/2
- Peninsula Laboratories, Inc., Belmont, CA, USA
  Biotin-CPG columns, Cat. No. N4039

E.2.2 Amino-modified DNA

Amino-modified nucleic acid fragments can be chemically biotinylated using a biotin-X-NHS ester™ (3, 5).

Recommended protocol for biotinylation of amino-modified DNA:
- 5' amino-modified oligo is synthesized using the reagent Aminolink 2™
  (Cat. No. 400808, Perkin Elmer - Applied Biosystems Division, Foster City, CA, USA).
- A biotin residue is attached to the amino group of the oligo using the biotin-X-ester reagent (incubate at room temperature overnight).

  Recommended biotin-X-NHS ester:
  - Boehringer Mannheim GmbH, Mannheim, Germany
    D-Biotin-N-hydroxysuccinimide ester, Product #732 494
  - Clontech Laboratories, Inc., Palo Alto, CA, USA
    Biotin-X-NHS Ester, Cat. No. 5002-1/2

- Unincorporated biotin-X-ester is removed with a NAP®-10 Column
  (Pharmacia Biotech AB, Uppsala, Sweden, Product #170854-01/02).
- The biotinylated oligo is purified by reverse phase HPLC/FPLC according to the manufacturer's description.

E.2.3 Larger DNA fragments

- Enzymatic incorporation of a biotin dUTP label
  A biotin dUTP label can be enzymatically incorporated into a double-stranded DNA fragment through end-labeling by use of Klenow DNA polymerase enzyme, nick translation or mixed primer labeling (6, 7).

  Recommended biotin dUTP:
  - Boehringer Mannheim GmbH, Mannheim, Germany
    Biotin-16-dUTP, Product #1093 070
  - Clontech Laboratories, Inc., Palo Alto, CA, USA
    Biotin-21-dUTP, Cat. No. 5021-1

- Photobiotinylation
  The photoactivated form of biotin can be randomly incorporated in the DNA fragment with UV light (8).

  Recommended photobiotin:

  - Life Technologies, Inc., Gaithersburg, MD, USA
    Photobiotin® Labeling System, Product #530-8186SA

- End-labeling using PCR with a biotinylated primer.

E.2.4 Cleavable biotin derivatives of DNA fragments

The high stability of the biotin/streptavidin bond prevents the bond from being easily split. Using a modified biotin analog, it is possible to dissociate the biotinylated DNA fragment from the Dynabeads M-280 Streptavidin.

- Enzymatic incorporation of a biotin dUTP analog with a cleavable linker.
  Incorporation of a biotin with a linker arm containing a disulfide bond allows for a simple dissociation of the DNA fragment, as the disulfide link is easily cleaved with dithiothreitol (DTT). This reagent is enzymatically incorporated into a DNA fragment either by end-labeling, nick translation or mixed primer labeling.
  Biotin-21-SS-dUTP is recommended.

- Chemical incorporation of the guanido analog of NHS-biotin
  (NHS-Iminobiotin, Product #21117, Pierce Chemical Corp., Rockford, IL, USA). Incorporation of iminobiotin allows for the dissociation of the bound nucleic acid fragment with a simple pH change. The streptavidin/iminobiotin complex is dissociated at pH 4.0. At pH 9.5 or greater, iminobiotin will bind tightly to Dynabeads M-280 Streptavidin. The released iminobiotin can be reimmobilized onto Dynabeads M-280 Streptavidin.

E.2.5 RNA fragments

In most cases, procedures for biotinylating DNA can be followed when biotinylating RNA.

Aminolink 2™ can be used to amino-modify the RNA fragment, following the same procedure for amino-modifying DNA. The amino-modified fragment can be chemically biotinylated using a biotin-X-NHS ester (see section E.2.2).

The Nonradioactive RNA Labeling System (Life Technologies, Inc., Gaithersburg, MD, USA, Product #8093SA) can be used to label RNA as it is transcribed from DNA.

RNA fragments that have already been synthesized can be photobiotinylated. As with DNA, the photoactivated form of biotin is randomly incorporated into the RNA fragment with UV light.

Recommended photobiotin:
- Life Technologies, Inc., Gaithersburg, MD, USA
  Photobiotin™ Labeling System, Product #530-8186SA

E.2.6 Purification of biotinylated primers

It is of great importance that the biotinylated oligonucleotide is purified from unbound biotin, preferably by FPLC® (10) or reverse phase HPLC (11), since free biotin will occupy binding sites on the beads and reduce the binding capacity of biotinylated PCR products. This purification step also ensures full length oligodeoxynucleotides with labeling levels close to 100%.

When performing the biotinylation of primers using biotin phosphoramidite, you can expect to obtain 70-75% biotinylation of your primer. This means that 25-30% of your primer may be non-biotinylated. Reverse phase HPLC or FPLC is necessary to recover the biotinylated primers in pure form.

Recommended protocol for purification of biotinylated primers by reverse phase HPLC chromatography:

Column: S5 ODS2 SPHERISORB 10 cm x 20 mm id
(Phase Separations Ltd., Deeside Clwyd, UK, Part No. 831992)

Mobile phases:
- Acetonitrile, Lab Scan Ltd., Dublin, Ireland, Code No. H6502
- 2 M Triethylamine Acetate, HPLC grade, Perkin Elmer - Applied Biosystems Division, Foster City, CA, USA, Cat No. 400613

A: 5% Acetonitrile in 0.1 M Triethylamine Acetate, pH 7.0
B: 40% Acetonitrile in 0.1 M Triethylamine Acetate, pH 7.0

Gradient:

0 min		95% A	5% B
0 - 12 min	linear increase to	57% A	43% B
12 - 20 min	linear increase to	0% A	100% B
20 - 30 min	hold at	0% A	100% B
30 - 35 min	linear increase to	95% A	5% B
35 - 45 min	hold at	95% A	5% B

Flow: 5.0 ml/min

Pressure: Approx. 1600 psi

Detector: UV detector at 270 nm

Sample: One complete 0.2 or 1.0 μmol synthesis, lyophilized and diluted to 1.0 ml in mobile phase A.

A synthesized, unpurified 5' biotinylated oligonucleotide primer will give two prominent and characteristic peaks on your reverse-phase HPLC chromatogram. The first of these peaks is the non-biotinylated oligo, while the second and the latter eluted peak is the 5' biotinylated oligo which will appear after approximately 15 to 20 minutes depending of the size of the primer. Start the collection of the second peak when it begins to increase. End the collection when the chromatogram decreases and begins to round out. It takes about 2 minutes and the collection volume will be approximately 10 ml. Collect two fractions and concentrate the solution by evaporation to 0.5 ml (for a 0.2 μmol synthesis), and load this sample onto a NAP-5 column (Pharmacia Biotech AB, Uppsala, Sweden) for a further purification and buffer changes.

The consequence of using a partially purified biotinylated primer in your PCR is obvious. A proportion of the unpurified biotinylated primer preparation will also result in PCR products, but without biotin in the 5' end. These PCR products cannot be bound to Dynabeads M-280 Streptavidin.
This effect has been simulated to show the effect of using poorly purified biotinylated primers. A purified biotinylated primer and a non-biotinylated primer containing exactly the same primer sequence have been mixed in different ratios and added to another primer for PCR. Figure E.1 shows the amount of PCR products bound to a constant amount of Dynabeads M-280 Streptavidin.

The non-biotinylated PCR products do not hinder the binding of the biotinylated products, so that it could in principle be possible to compensate for this effect by using different amounts of primers. However, the results would be difficult to predict, and involve additional work by the user. To ensure reliable and predictable results, Dynal **strongly recommends** using only purified primers.

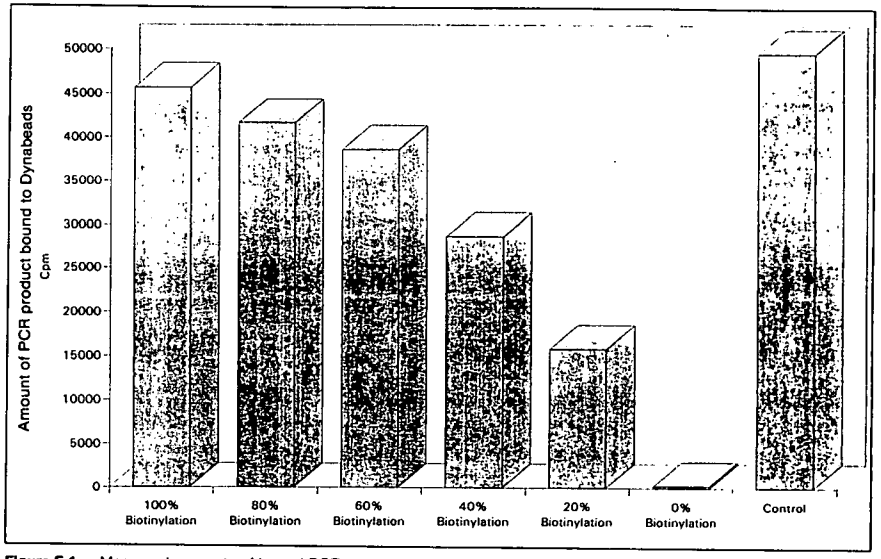


Figure E.1 Measured amounts of bound PCR products per 200 μg (20 μl) Dynabeads M-280 Streptavidin with different ratios between the same purified biotinylated primer and non-biotinylated primer (5:0, 4:1, 3:2, 2:3, 1:4, 0:5) using ^{33}P-α-dATP in the PCR reaction mix and 5 pmoles of each PCR primer.

E.3 Biotinylation of proteins

E.3.1 Proteins

Proteins can be chemically biotinylated using a biotin-X-NHS ester (9).

Recommended biotin-X-NHS ester:
- Pierce Chemical Corp., Rockford, IL, USA
  NHS-LC-Biotin, Product #21335

E.3.2 Cleavable biotin derivatives of proteins

NHS-biotin containing a cleavable disulfide bond allows for the easy cleavage of the desired protein from the biotin/streptavidin complex (12) (see section E.2.4).

Recommended NHS-SS-Biotin:
- Pierce Chemical Corp., Rockford, IL, USA
  NHS-SS-Biotin, Product #21331

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F. IMMOBILIZATION OF BIOTINYLATED NUCLEIC ACIDS TO DYNABEADS M-280 STREPTAVIDIN

F.1 General introduction

Dynabeads M-280 Streptavidin can be used for direct immobilization of biotinylated compounds such as double-stranded DNA fragments (PCR products), single-stranded DNA fragments or RNA fragments, proteins, sugars and lectins. The immobilization is essential for carrying out the solid-phase applications described in the first part of this technical handbook.

The stability and strength of the biotin-streptavidin interaction (K_d=10^{-15} M) allows DNA manipulations, such as strand melting, elution and hybridization (using alkali, temperature or formamide) to be performed without interfering with the immobilization of the DNA on the beads.

Applications include: solid-phase DNA sequencing, DNA/RNA capture by hybridization, labeling of single-stranded nucleic acid probes, gene assembly, *in vitro* mutagenesis, YAC-screening, DNA cloning and sequence-specific purification of DNA/RNA binding proteins.

This section gives you a description how to perform the immobilization of biotinylated nucleic acids to Dynabeads M-280 Streptavidin. For each specific application, more details are given in the respective chapters.

F.2 Materials required

- Dynabeads M-280 Streptavidin
- Biotinylated single- or double-stranded nucleic acids
- Dynal Magnetic Particle Concentrator (Dynal MPC)
- PBS buffer
- Binding & Washing buffer (B&W buffer)
- Rotator, if longer fragments are immobilized (> 1,0 kb)

F.3 Protocol

1. Wash the Dynabeads M-280 Streptavidin once in the B&W buffer. Washing is performed by a repeated collecting of the beads on the inside wall of the tube using a Dynal MPC, removal of the previous buffer by aspiration (while the tube is still placed in the Dynal MPC) and resuspension of the Dynabeads M-280 Streptavidin in the buffer for the next step. For detailed washing protocol see appendix B. The beads can be washed in bulk.
2. If Dynabeads have been washed in bulk, aliquot in Eppendorf tubes or microtiter wells and remove the buffer from the last washing step.
3. Resuspend the beads in B&W buffer to a final concentration of 5 µg/µl (twice the original volume), or to a concentration suitable for the application of choice.
4. Add an equal volume of the biotinylated DNA/RNA to be immobilized. The NaCl concentration in the B&W buffer is 2 M and the final NaCl concentration in the binding mixture should be 1 M.
   The amount of DNA/RNA needed is dependent on the application, please see the chapter covering the application to be run, or the capacity table below (the capacity is dependent on the DNA fragment length).
5. Incubate at room temperature using gentle rotation or occasional mixing by gently tapping of the tubes. The optimal incubation time depends on the length of the nucleic acid to be bound: short oligonucleotides (less than 30 bases) require at most 10 minutes. DNA frag-

ments up to 1 kb require 15 minutes. For DNA fragments longer than 1 kb, we recommend using a longer incubation time (15–60 minutes) at 43°C. Alternatively, the binding can be improved by overnight incubation at 25°C (see note under F.4).

> **Note:** The efficiency of the binding, is influenced by salt concentration. Extensive experience has shown that for optimal binding, a salt concentration of 1 M is required in the binding buffer.

6. Separate the Dynabeads M-280 Streptavidin, now coated with the biotinylated DNA/RNA fragment, using a Dynal MPC. Leave the tube/tray in the Dynal MPC for 1 to 2 minutes.
7. Wash 2-3 times with B&W buffer, using a Dynal MPC as described above in point 1.
8. Resuspend to the desired concentration. The binding is now completed and the Dynabeads with the immobilized DNA/RNA fragment can be resuspended in a buffer with lower salt concentration, suitable for downstream applications.

F.4 Technical tips

Binding capacity
The binding capacity of Dynabeads M-280 Streptavidin is fragment length dependent. Quantitative assays of ^{32}P-labeled PCR product binding to beads, show the effect of the length of the PCR product on binding. Twice as many copies of a 500 bp DNA fragment bind to the Dynabeads M-280 Streptavidin than a 1000 bp DNA fragment (see Fig F.1). Reduced binding capacity for large DNA fragments may be caused by steric hindrance.

Optimal binding conditions for biotinylated DNA fragments onto Dynabeads M-280 Streptavidin:

Up to 1 kb	1 M NaCl (final concentration) 25°C 15 minutes
Above 1 kb	1 M NaCl or 3 M LiCl (final concentration) 43°C Extend the incubation time (15–60 minutes), alternatively overnight incubation at 25°C

DNA fragments of 6,2 kb has routinely been coupled to Dynabeads M-280 Streptavidin with a binding capacity of 1,0 pmole/mg Dynabeads (4,2 µg/mg Dynabeads) by Sandaltzopoulos *et al.* (1). In this case the couplings were done overnight at 25°C on a rotating wheel.

Because free biotin and free biotinylated oligonucleotides (not used during a PCR amplification) bind to the Dynabeads M-280 Streptavidin much more rapidly than longer PCR fragments, **it is most import to ensure that the solution containing the PCR fragments does not contain an excess of these components.** To prevent an excess of free biotinylated oligonucleotides, one may perform PCR with limiting concentrations of the biotinylated primer, or remove the free biotinylated primer via precipitation or by microdialysis.

Salt concentration
Extensive experience has shown that for optimal binding, a salt concentration of 1 M is required in the binding buffer.

Biotinylation of primers/fragments
Proper biotinylation of the PCR primers and restricted DNA fragments is of course a requirement for immobilization of the nucleic acids to the Dynabeads M-280 Streptavidin. Non HPLC purified biotinylated primers often contain considerable amounts of non-biotinylated primers.

PCR products generated with such primers will contain a fraction which is unable to bind to the Dynabeads M-280 Streptavidin. The proportion of the PCR products unable to bind will be the same as the proportion of non-biotinylated primers in the primer preparation (i.e. there is no discrimination between biotinylated and non-biotinylated primers during PCR). For biotinylation procedures, see Appendix E.

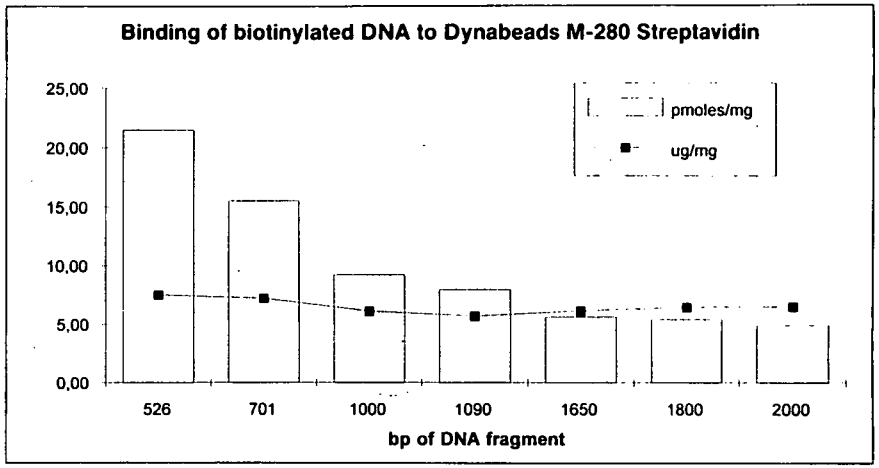

Figure F.1 The binding capacity of Dynabeads M-280 Streptavidin as a function of the DNA fragment length (in pmoles/mg and in µg/mg).

F.5 Buffers and solutions

Phosphate-buffered saline, pH 7.5 (PBS buffer)	137 mM NaCl 2.7 mM KCl 4.3 mM $Na_2HPO_4 \cdot 7H_2O$ 1.4 mM KH_2PO_4
Binding & Washing Buffer (B&W buffer)	10 mM Tris-HCl, pH 7.5 1 mM EDTA 2.0 M NaCl (final concentration: 1.0 M)

F.6 References

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G. DISSOCIATION OF BIOTINYLATED DNA FROM DYNABEADS M-280 STREPTAVIDIN

G.1 General introduction

The biotin/streptavidin complex can withstand various "harsh" conditions without dissociation of the biotin-streptavidin link. In some cases however it is desirable to break the biotin-streptavidin interaction to release the target material from the Dynabeads. Some possible dissociation methods are as follows.

G.2 Recommendations

G.2.1 Heating in formamide solution (1)

Biotinylated single-stranded DNA fragments have been successfully dissociated from Dynabeads M-280 Streptavidin by heating the complex for at least 2 minutes at a minimum temperature of 65°C in a buffer containing 10 mM EDTA, pH 8.2, and 95% formamide. Elution efficiencies higher than 96% have been reported.

> **Note:** It is important to remove the Dynabeads M-280 Streptavidin from the supernatant while the supernatant is still hot. This will prevent reannealing of the biotinylated strands to the Dynabeads.

G.2.2 Phenol treatment (2)

The biotinylated DNA fragments have been removed from the Dynabeads M-280 Streptavidin by incubating the complex for 30 minutes at 65°C in 50% (v/v) phenol.

G.2.3 Incorporation of a cleavable linker arm linked to the biotin molecule

Using a modified biotin analog, it is possible to dissociate the biotinylated DNA fragment from the Dynabeads M-280 Streptavidin.

1. *Enzymatic incorporation of a biotin dUTP analog*
   Incorporation of a biotin with a linker arm containing a disulfide bond allows the simple dissociation of the DNA fragment, as the disulfide link is easily cleaved with dithiothreitol (DTT). This reagent is enzymatically incorporated into a DNA fragment either by end-labeling, nick translation or mixed primer labeling.
2. *Chemical incorporation of the guanido analog of NHS-biotin.*
   Incorporation of iminobiotin allows for the dissociation of the bound DNA with a simple pH change. The streptavidin/iminobiotin complex is dissociated at pH 4.0. At pH 9.5 or greater, iminobiotin will bind tightly to Dynabeads M-280 Streptavidin.

   See section E.2.4 for recommended reagent.

G.2.4 Digestion of the immobilized DNA fragments with restriction enzymes (2-4)

It has been reported that the 5' end of a biotinylated oligonucleotide primer has been designed to include a restriction site to facilitate digestion with unique restriction enzymes for detachment (2, 3). This may allow later directional cloning by "sticky-end" ligation.

In some cases, a restriction enzyme can also be used to cleave the desired DNA between the two primers for restriction site mapping (4). The DNA fragment used as well as the restriction enzyme chosen, will determine the portion of the fragment cleaved from the beads.

G.2.5 Boiling with SDS

Unpublished data suggests that it is possible to dissociate the biotinylated DNA fragment from the Dynabeads M-280 Streptavidin by boiling the complex for 5 minutes in 0.1% SDS.

> **Note:** It is important to remove the Dynabeads M-280 Streptavidin from the supernatant while the supernatant is still hot. This will prevent reannealing of the biotinylated strands to the Dynabeads.

G.3 References

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PRECAUTIONS

Thoroughly resuspend the Dynabeads M-280 products before use to obtain a homogenous dispersion of beads in solution. Caution should be taken to prevent RNase contamination of Dynabeads Oligo $(dT)_{25}$ and the kit components belonging to this product. For all the other products, caution should be taken to prevent microbial contamination.

Preservatives should carefully be removed before use by washing as described in Appendix D, preparation of Dynabeads M-280.

WARNINGS

Dynabeads Oligo $(dT)_{25}$, Dynabeads M-280 Streptavidin and Dynabeads lacZ contain 0.02% sodium azide (NaN_3) as a preservative. Sodium azide may react with lead and copper drain-pipes to form highly explosive metal azides. When disposing through drains, flush with large volumes of water to prevent azide buildup. Sodium azide is toxic if ingested. **Avoid pipetting by mouth.**

The reconditioning solutions in the Dynabeads lacZ ssM13 Purification Kit and in the Dynabeads mRNA DIRECT Kit contain 0.5 M NaOH and 0.1 M NaOH, respectively. **Can cause burns of eyes and skin. May cause coughing, difficulty breathing, lung damage, diarrhea or shock if inhaled or ingested. Read Material Safety Data Sheet (MSDS).**

Hybridization solution in the Dynabeads lacZ ssM13 Purification Kit contains 2.5 M $NaClO_4$ and 12% polyethylene glycol (PEG). **May cause eye and skin irritation. Do not store with acids and bases. Read MSDS.**

Certificates of Analysis (CoA) and MSDS on solutions with Dynabeads and ingredients that can be hazardous in use, may be obtained on request.

The Dynal MPCs should not be kept in close contact with magnetic tapes, computer discs or other magnetic storage systems, as these can be damaged by the strong magnetic field.

LIMITATIONS

The Dynal products described in this technical handbook are for research use only. The products are not for use in human diagnostic or therapeutic procedures.
The Dynal products may not be repacked, reformulated or resold in any form without written consent of Dynal A.S, Oslo, Norway.
See also references to current patents on page 159.

WARRANTY

The products are warranted to the original purchaser only to conform to the specifications indicated on the unit label for a period of one year from the date of purchase. DYNAL's obligation and the purchaser's exclusive remedy under this warranty is limited either to replacement, at DYNAL's expense, of any products which shall be defective in manufacture, and which shall be returned to DYNAL, transportation prepaid, or at DYNAL's option, refund of the purchase price. It shall be the responsibility of the purchaser to pack returned item(s) in a manner to avoid shipping damage to the unit.

Claims for merchandise damaged in transit must be submitted to the carrier.

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Aminolink 2™	Perkin Elmer - Applied Biosystems Division
AmpliTaq®	Hoffmann-La Roche, Inc.
Biodite™	Pharmacia Biotech AB
Biomek®	Beckman Instruments, Inc.
Biotin-ON™	Clontech Laboratories, Inc.
Biotin-X-NHS ester™	Clontech Laboratories, Inc.
Catalyst™	Perkin Elmer - Applied Biosystems Division
Centricon®	Amicon, Inc.
DETACHaBEAD®	Dynal A.S
DIANA®	Dynal A.S
Duall®	Kontes Biotechnology
Dynabeads®	Dynal A.S
Dynal®	Dynal A.S
Dynal MPC®	Dynal A.S
Eppendorf®	Eppendorf-Netheler-Hinz GmbH
FPLC®	Pharmacia Biotech AB
GeneAmp®	Hoffmann-La Roche, Inc.
Lambda ZAP®	Stratagene Cloning Systems
mRNA DIRECT™	Dynal A.S
NAP®	Pharmacia Biotech AB
Nonidet®	Shell Oil Company
pBluescript®	Stratagene Cloning Systems
pCR®	Invitrogen Corporation
pGEM®	Promega Corporation
Pellet Pestle®	Kontes Biotechnology
Photobiotin®	Biotechn. Res. Ent. S.A. Pty. Ltd.
PolySeq™	Polygen Corporation
Polytron®	Kinematica AG
PRISM™	Perkin Elmer - Applied Biosystems Division
QPCR™	Perkin Elmer - Applied Biosystems Division
RNAguard®	Pharmacia Biotech AB
RNasin®	Promega Corporation
Sephadex®	Pharmacia Biotech AB
Sequenase®	U.S. Biochemical Corporation
TA Cloning®	Invitrogen Corporation
Tris®	Rohm & Haas, Inc.
Triton®	Rohm & Haas, Inc.
Tween®	ICI Americas, Inc.
USB™	U.S. Biochemical Corporation
Vent®	New England Biolabs, Inc.

Patents:

Taq DNA Polymerase, AmpliTaq DNA Polymerase and the GeneAmp PCR Process is the subject of patents and patent applications of Hoffmann-La Roche, Inc., USA

The Dynal products are covered by various international patents and/or patent applications.

ORDERING INFORMATION

Please provide the following information when ordering:

1 Shipping address
2 Invoice address (with telephone and fax number)
3 Product number
4 Product description
5 Quantity and size
6 Purchase order number

Returns
It is our policy to deny return of product. Due to the temperature sensitivity of the Dynabeads and the other kit components, we are unable to resell returned goods, as we are not assured of the quality.

Product use limitations
All products are for research purpose only, not for drug or diagnostic use unless otherwise indicated. The products are not for resale without written concent from Dynal A.S, Oslo, Norway.

PRODUCTS FOR MOLECULAR BIOLOGY

	Prod. No.	Volume
Dynabeads mRNA DIRECT Kit	610.11	1 kit
Dynabeads mRNA Purification Kit	610.01	1 kit
Dynabeads Oligo $(dT)_{25}$	610.02	2x1 ml
	610.05	5x1 ml
Dynabeads Template Preparation Starter Kit	612.02	1 kit
Dynabeads Template Preparation Kit	612.01	1 kit
Dynabeads M-280 Streptavidin	112.05	2 ml
	112.06	10 ml
Dynabeads lacZ ssM13 Purification Kit	625.01	1 kit
Dynabeads lacZ	625.06	10 ml
Dynal MPC-1	120.01	Piece
Dynal MPC-P-12	120.10	Piece
Dynal MPC-E-1	120.07	Piece
Dynal MPC-E	120.04	Piece
Dynal MPC-M	120.09	Piece
Dynal MPC-96	120.05	Piece
Dynal MPC-9600	120.06	Piece